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13. ABSTRACT (Maximum 200 Words)

TR3 (also called Nur77 or NGFI-B), an immediate-early response gene and an orphan member of the nuclear receptor superfamily, regulates both survival and death of prostate cancer cells. It is overexpressed in prostate tumor and acts in the nucleus to promote tumor cell growth. In response to certain apoptotic stimuli, TR3 migrates from the nucleus to the cytoplasm where it targets mitochondria to induce cytochrome *c* release and apoptosis. Translocation of TR3 from the nucleus to the cytoplasm requires its heterodimerization with retinoid X receptor (RXR) and it is highly regulated by RXR ligands. In addition, phosphorylation of TR3 protein modulates its subcellular localization and biological activities. Mitochondrial localization of TR3 is mediated by its interaction with Bcl-2, a potent anti-apoptotic protein that is overexpressed in prostate tumors. Binding of TR3 to Bcl-2 induces a Bcl-2 conformational change, resulting in conversion of Bcl-2 from a protector to killer. These results establish a novel TR3-dependent apoptotic pathway in prostate cancer cells and its regulation. They also demonstrate that TR3 is an attractive molecular target for developing new prostate cancer therapeutics.

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INTRODUCTION

Cancer of the prostate gland with 30% incidence is the primary cancer of American men and their second leading cause of cancer death. These statistics show the necessity for more effective treatment. Recent progress has suggested that induction of cancer cell death is a plausible way to restrict tumor growth, and many chemotherapeutic drugs induce death of cancer cell. Knowing how prostate cancer cell apoptosis is regulated would offer a rational guide for developing agents for preventing and treating this disease.

Human testicular receptor 3 (TR3, also known as nur77 or NGFI-B) is an orphan member of the nuclear receptor (NR) superfamily that is strongly induced by androgens in prostate cancer cells (Maruyama et al., 1998; Zhang, 2002) and highly expressed in prostate tumor compared to adjacent normal or benign prostate hypertrophic tissue (Uemura and Chang, 1998). We recently discovered a novel apoptotic (programmed cell death) pathway in prostate cancer cells in which TR3 migrates from the nucleus to mitochondria to initiate apoptosis (Li et al., 2000; Zhang, 2002). Apoptosis-associated nuclear egress of TR3 has also been observed in cancer cells from breast, gastric system, lung, or ovary (Dawson et al., 2001; Holmes et al., 2002; Holmes et al., 2003; Jeong et al., 2003; Kolluri et al., 2003; Lee et al., 2002; Lin et al., 2004; Wilson et al., 2003; Wu et al., 2002). The importance of this pathway in mediating anticancer activity is supported by the positive correlation between TR3 family member Nor-1 expression and survival in diffuse large B-cell lymphoma patients on chemotherapy (Shipp et al., 2002)and a recent observation that TR3 is one of the 17-gene signature associated with metastasis of primary solid tumors, including prostate tumor (Ramaswamy et al., 2003).

This application focuses on the molecular mechanism by which TR3 targets mitochondria and induces apoptosis of prostate cancer cells. With the funding from PCRP, we have made significant advances towards our understanding of the TR3-dependent apoptotic pathway in prostate cancer cells and its regulation.

KEY RESEARCH ACCOMPLISHMENTS

Identification of TR3 mitochondrial receptor (***Cell*, 116,527-540, 2004**). TR3 exerts its apoptotic effect by targeting mitochondria. One of the goals of this application is to determine how TR3 targets mitochondria. TR3 does not have a classical mitochondrial targeting sequences. We proposed that it might target mitochondria through its interaction with mitochondrial protein. Members of the Bcl-2 family are important regulators of cell death and survival (Reed, 1998). Many of which, such as Bcl-2, are located predominantly in the mitochondrial outer membrane. We investigated the possibility that TR3 targeted mitochondria by interacting with Bcl-2. By using a variety of approaches, including GST-pull-down, mammalian two-hybrid studies, reported gene assay, and immunoprecipitation assays, we found that TR3 physically interacts with Bcl-2 (Lin et al., 2004). To study whether TR3 interacted with Bcl-2 in LNCaP prostate cancer cells, we generated a monoclonal antibody against the ligand-binding domain (LBD) of TR3 for co-immunoprecipitation (Co-IP) assay. LNCaP cells were treated with phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which induces expression of endogenous TR3 and its mitochondrial localization in LNCaP prostate cancer cells (Li et al., 2000). Cells extracts were prepared from TPA-treated and nontreated cells and incubated with anti-TR3 antibody. Bcl-2 was specifically coprecipitated by anti-TR3 antibody in TPA-treated cells, but not in nontreated cells. To determine whether endogenous TR3 and Bcl-2 colocalized in prostate cancer cells, we conducted confocal microscopy analysis. Our results demonstrated that, while low level of TR3 was detected in the nucleus of LNCaP cells in the

absence of TPA, the distribution patterns of TPA-induced TR3 and Bcl-2 overlapped extensively in the cytoplasm (Lin et al., 2004). Together, these data demonstrate that Bcl-2 acts as a TR3 mitochondrial receptor (Figure 2).

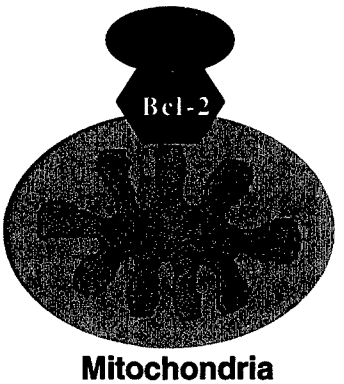

Figure 1. Bcl-2 acts as a mitochondrial receptor for TR3. Bcl-2, which mainly resides on outer mitochondrial membrane, mediates TR3 mitochondrial localization through its interaction with TR3.

A Unique TR3/Bcl-2 interaction (*Cell*, 116,527-540, 2004)

Three-dimensional structures of Bcl-2 family members demonstrate the BH1, BH2, and BH3 regions form an elongated hydrophobic cleft, to which a BH3 amphipathic α helix binds (Petros et al., 2001; Sattler et al., 1997). However, deletion or point mutations in the hydrophobic cleft did not affect the interaction between TR3 and Bcl-2 (Lin et al., 2004), indicating that the BH3-binding hydrophobic groove in Bcl-2 is not involved in binding TR3. These results suggested that the N-terminal portion of Bcl-2 was responsible for binding TR3. Therefore, we constructed a mutant of Bcl-2, which contains the N-terminal 80 amino acid residues (Bcl-2/1-80). Our co-immunoprecipitation (Co-IP) assay showed that Bcl-2/1-80 could interact with TR3/ΔDBD, similar to the full-length Bcl-2 (Lin et al., 2004). The Bcl-2/1-80 encompasses the N-terminal BH4 domain and an unstructured loop domain of approximately 50 amino acid residues in length. To determine whether the BH4 domain or the loop region was responsible for binding TR3/ΔDBD, we investigated the interaction of TR3/ΔDBD with Bcl-2 mutants lacking either the BH4 domain (Bcl-2/ΔBH4) or the loop region (Bcl-2/ΔLoop) (Lin et al., 2004). Our Co-IP studies demonstrated that the Bcl-2 mutant lacking the BH4 domain retained the ability to interact with TR3/ΔDBD. In contrast, deletion of the loop region from Bcl-2 completely abolished its ability to bind TR3/ΔDBD (Lin et al., 2004). Thus, these results demonstrate that the loop region in Bcl-2 is responsible for binding TR3.

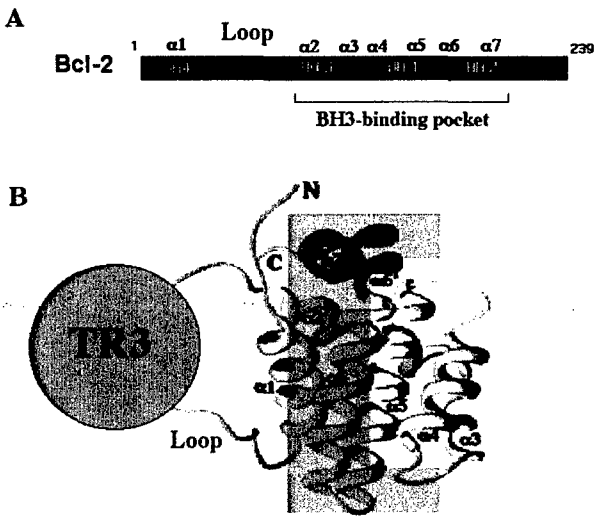

Figure 2. The loop domain of Bcl-2 is responsible for binding TR3. **(A)** Schematic representation of Bcl-2 protein. BH: Bcl-2 homolog domain. BH and loop domains as well as a-helix are indicated. **(B)** Schematic representation of binding of TR3 to the Bcl-2 loop region.

Bcl-2 is required for TR3 induction of cytochrome c release and apoptosis (*Cell*, 116,527-540, 2004).

TR3 mitochondrial targeting is essential for TR3 induction of cytochrome c release and apoptosis (Li et al., 2000). Our observation that Bcl-2, a potent apoptosis inhibitor, was required for mitochondrial targeting by TR3 suggested that Bcl-2 was needed for apoptosis. This appeared to be contradictory. We examined the requirement of TR3 interaction with Bcl-2 for TR3-induced cyt *c* release and apoptosis. By using confocal microscopy, biochemical analysis, and apoptosis assays, we found that Bcl-2 expression was required for induction of cytochrome c release and apoptosis by TR3, whereas inhibition of Bcl-2 expression by Bcl-2 siRNA or Bcl-2 dominant-negative mutant abolished TR3-dependent apoptosis (Lin et al., 2004). The pro-apoptotic effect of Bcl-2 seen upon co-expression was specific to TR3, because Bax-induced apoptosis was effectively prevented by Bcl-2 co-expression. Thus, Bcl-2 can manifest a pro-apoptotic phenotype in settings where TR3 is expressed and targets to mitochondria. In contrast, Bcl-2 suppresses apoptosis when co-expressed with Bax.

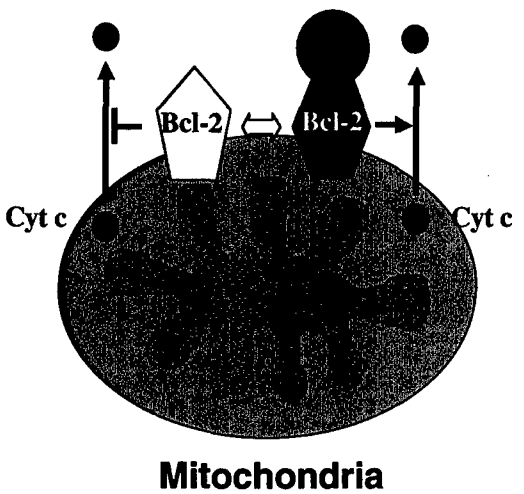

Figure 3. TR3 interaction with Bcl-2 induces cytochrome c release. Bcl-2 expression suppresses cytochrome c release. In contrast, interaction of Bcl-2 with TR3 results in extensive cytochrome c release and apoptosis.

The BH3 domain of Bcl-2 is critical for apoptosis induced by the TR3/Bcl-2 interaction (*Cell*, 116,527-540, 2004)

We have studied the possible mechanism by which Bcl-2, which is widely considered as a potent antiapoptotic molecule, acted as a proapoptotic molecule to mediate the proapoptotic effect of TR3. For this purpose, we constructed a number of TR3 and Bcl-2 mutants (Figure 4a), and analyzed their apoptotic effect. When TR3 mutants were analyzed, we observed that

coexpression of Bcl-2 with TR3/ΔDBD, DC3 or DC1 strongly induced apoptosis (Figure 4c). Thus, the minimal C-terminal domain of TR3, capable of binding to Bcl-2, was sufficient to induce apoptosis when it was coexpressed with Bcl-2. The requirement of the TR3/Bcl-2 interaction for inducing apoptosis was illustrated by the observation that mutants of TR3 (TR3/ΔDBD/ΔDC1, TR3/ΔDBD/Δ471-481, TR3/ΔDBD/L487A or TR3/ΔDBD/I483A), which failed to bind Bcl-2, did not induce apoptosis when they were coexpressed with Bcl-2 (Figure 4c). Our results demonstrated that the apoptotic function of Bcl-2 in the context of TR3/ΔDBD expression was completely abolished in Bcl-2 mutants Bcl-2/1-80, Bcl-2/ΔBH1, and Bcl-2/ΔBH3, despite their ability to interact with TR3/ΔDBD. These data demonstrate that TR3 binding, although essential, is not sufficient to induce apoptosis. The fact that Bcl-2/ΔBH4 was capable of inducing apoptosis in the presence of TR3/ΔDBD suggested the involvement of the C-terminal Bcl-2 sequences in mediating apoptosis induced by the TR3/Bcl-2 interaction. We then studied several Bcl-2 point mutants in this region. Bcl-2/L137A and Bcl-2/G145A, which failed to suppress the apoptotic effect of Bax, retained the ability to induce apoptosis when TR3/ΔDBD was cotransfected (Figure 3d). In contrast, Bcl-2/Y108K, which harbors a mutation in the BH3 domain, did not. Thus, an intact hydrophobic groove in Bcl-2 is required for its antiapoptotic effect but not its proapoptotic effect, whereas the BH3 domain of Bcl-2 is critical for its proapoptotic action.

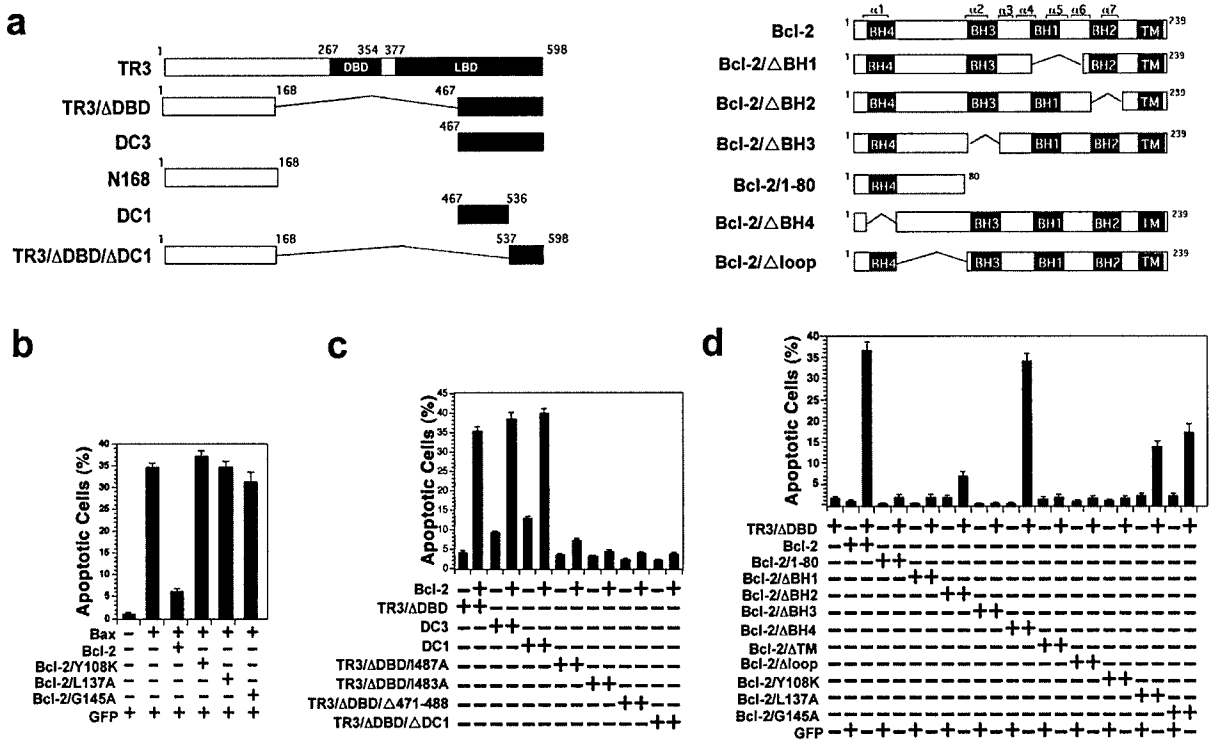

Figure 4. Apoptotic effects of TR3 and Bcl-2 mutants. (a). Schematic representation of TR3 and Bcl-2 mutants. DNA-binding domain (DBD) and ligand-binding domain (LBD) of TR3, and BH domains and a-helix of Bcl-2 are indicated. **(b).** Effect of Bcl-2 mutations on apoptotic effect of Bax. HEK293T cells were transfected with the indicated expression vectors. After 36 h, apoptotic cells were determined by DAPI and scored by examining 300 GFP-positive cells for nuclear fragmentation and/or chromatin condensation. **(c).** Interaction of TR3 with Bcl-2 is required for apoptotic effect of TR3/Bcl-2 co-expression. The indicated TR3 mutant was transfected with empty or Bcl-2 expression vector into HEK293T cells and apoptotic cells were

scored as described above. **(d).** Effect of Bcl-2 mutations on apoptotic effect of TR3/Bcl-2 co-expression. The indicated Bcl-2 or its mutant was transfected with GFP or GFP-TR3/ΔDBD into HEK293T cells. Cells were then stained by DAPI and apoptotic cells were scored as described above. The bars in **b-d** are means ± S.D. from three experiments.

Bcl-2 undergoes a conformational change upon TR3 binding (*Cell*, 116,527-540, 2004)

Bcl-2 promotes apoptosis when co-expressed with TR3 but suppresses apoptosis when co-expressed with Bax. Bcl-2 has a hydrophobic crevice (BH3-binding pocket) on its surfaces that bind the BH3 domains of other family members (Petros et al., 2001; Sattler et al., 1997). The BH3-binding pocket is essential for anti-apoptotic function of Bcl-2. Our analysis of structure–function relationships for the pro-apoptotic effect of Bcl-2 in TR3-induced apoptosis showed that an intact hydrophobic groove in Bcl-2 is required for its anti-apoptotic activity but not for its pro-apoptotic activity, demonstrating a structural distinction between these two opposing phenotypes of Bcl-2. We explored whether a conformational change might be involved in converting Bcl-2 function from anti-apoptotic to pro-apoptotic. By comparing the effects of TR3 on binding of Bcl-2 to various anti-Bcl-2 antibodies that recognize different epitopes using flow cytometry and immunoprecipitation, we found that TR3 binding induces a Bcl-2 conformational change that exposes its BH3 domain. Further analysis showed that such a conformational change is responsible for pro-apoptotic effect of Bcl-2.

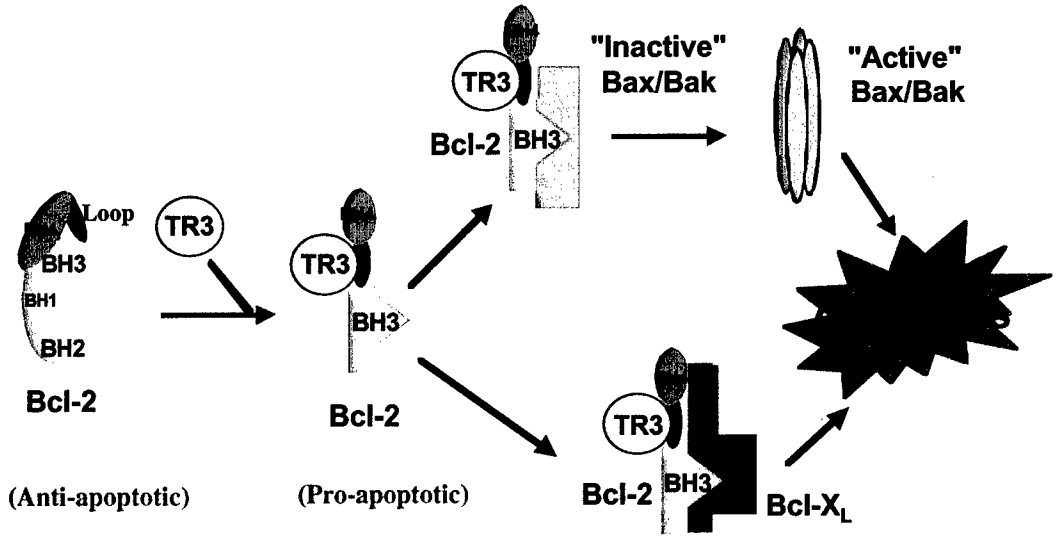

Figure 5. Binding of TR3 induces a Bcl-2 conformational change. Bcl-2 acts as an anti-apoptotic molecule through its BH3-binding pocket formed by BH3, BH2, and BH1 domains. However, when TR3 binds to the Bcl-2 loop region, Bcl-2 undergoes a reorganization of its BH3-binding pocket, resulting in exposure of its BH3 domain, which induces apoptosis by either binding to Bax/Bak to induce their oligomerization or binding to Bcl-XL to suppress its anti-apoptotic function.

Regulation of TR3 nuclear export ((*Mol. Cell. Biol.* 23, 8651-8667, 2003).

A number of protein kinase signaling pathways have been implicated in the regulation of nuclear receptor signalings. In this study, we demonstrated that TR3 expression was induced by epidermal growth factor and serum in lung cancer cells. However, unlike its induction by apoptotic stimuli, TR3 induced by growth factor and serum remains in the nucleus. Ectopic expressed TR3 also resides in the nucleus and it stimulated their cell cycle progression and proliferation, while inhibition of endogenous TR3 expression suppressed proliferation induced by growth factors. The mitogenic effect of TR3 required its DNA binding and transactivation functions, while they were dispensable for its apoptotic effect. Importantly, our results demonstrate that TR3 exerts opposing biological activities, survival and death, in the same cell type, depending on its subcellular localization. Furthermore, we found that MEKK1 strongly inhibited the transactivation and mitogenic effects of TR3 through activation of JNK that phosphorylated TR3, resulting in loss of its DNA binding activity.

REPORTABLE OUTCOMES

Zhang, X.-k. Vitamin A and apoptosis in prostate cancer. Endocrine-Related Cancer. 9: 87-102. 2002.

Kolluri, S., Cao, X., Bruey-Sedano, N., Lin, B., Lin, F., Han, Y.-H., Dawson, M.I., and Zhang, X.k. Mitogenic Effect of Orphan Receptor TR3 and its Regulation by MEKK1 in Lung Cancer Cells. *Mol. Cell. Biol.* 23: 8651-8667 2003.

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CONCLUSIONS

TR3, which is strongly induced by androgens in prostate cancer cells and highly expressed in prostate tumor compared to adjacent normal or benign prostate hypertrophic tissue (Uemura and Chang, 1998), translocates from the nucleus to mitochondria to trigger cytochrome *c* release and apoptosis of prostate cancer cells. Our results demonstrate that Bcl-2, with its ability to interact with TR3, functions as a mitochondrial receptor of TR3 to mediate TR3 mitochondrial targeting and its apoptotic effects. Our finding that TR3 interacts with Bcl-2 represents the first example that a nuclear receptor interacts with a Bcl-2 family member,

providing the coupling of TR3 nuclear receptor signaling to the Bcl-2-mediated apoptotic machinery.

Our findings that Bcl-2 acts as a bridging factor allowing TR3 mitochondrial localization and apoptosis indicate that Bcl-2 does not act as an antiapoptotic protein in TR3-mediated apoptosis. Our results demonstrate that the interaction between TR3 and Bcl-2 triggers cytochrome *c* release and apoptosis. Thus, the TR3/Bcl-2 interaction converts Bcl-2 from an antiapoptotic to a proapoptotic molecule, providing a new approach to provoke proapoptotic activity of Bcl-2. Our finding that the loop domain of Bcl-2 binds TR3 reveals a new regulatory mechanism by which a proapoptotic protein modulates the Bcl-2 function.

Our analysis showed that the hydrophobic groove of Bcl-2 underwent an extensive conformational change upon TR3 binding, resulting in exposure of its BH3 domain. Such a conformational change may be responsible for the conversion of Bcl-2 from an antiapoptotic to a proapoptotic molecule. Therefore, our results suggest that Bcl-2, upon TR3 binding, acts as a proapoptotic molecule by either binding to Bak to induce its oligomerization or binding to Bcl-XL to antagonize its antiapoptotic function.

Bcl-2 is often overexpressed in cancer cells and is associated with multidrug resistance. The finding that TR3 uses Bcl-2 as a receptor to target mitochondria and induce cell death suggests that molecules or pathways that induce apoptosis via TR3 have therapeutic potential. Thus, our results further validate that TR3 represents an ideal molecular target for developing cancer therapeutic drugs targeting Bcl-2-overexpressing cancer cells. Small molecule therapeutics that mimic the Bcl-2 binding domain of TR3 may also be selectively effective against Bcl-2-over-expressing cancer cells.

In conclusion, our results, together with recent clinical observations that TR3 family member Nor-1 expression is positively correlated with the survival in diffuse large B-cell lymphoma patients on chemotherapy (Shipp et al., 2002) and that TR3 is one of the 17-gene signature associated with metastasis of primary solid tumors, including prostate tumor (Ramaswamy et al., 2003), indicate that TR3 is an attractive molecule for developing novel therapeutics for prostate cancer.

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APPENDICES

Lin, B., Kolluri, S., Cao, X., Li, H., Han, Y.-h., Lin, F., Reed, J.C., and Zhang, X.-k. Conversion of Bcl-2 from Protector to Killer by Interaction with Nuclear Orphan Receptor Nur77/TR3. *Cell.* 116: 1-20. 2004.

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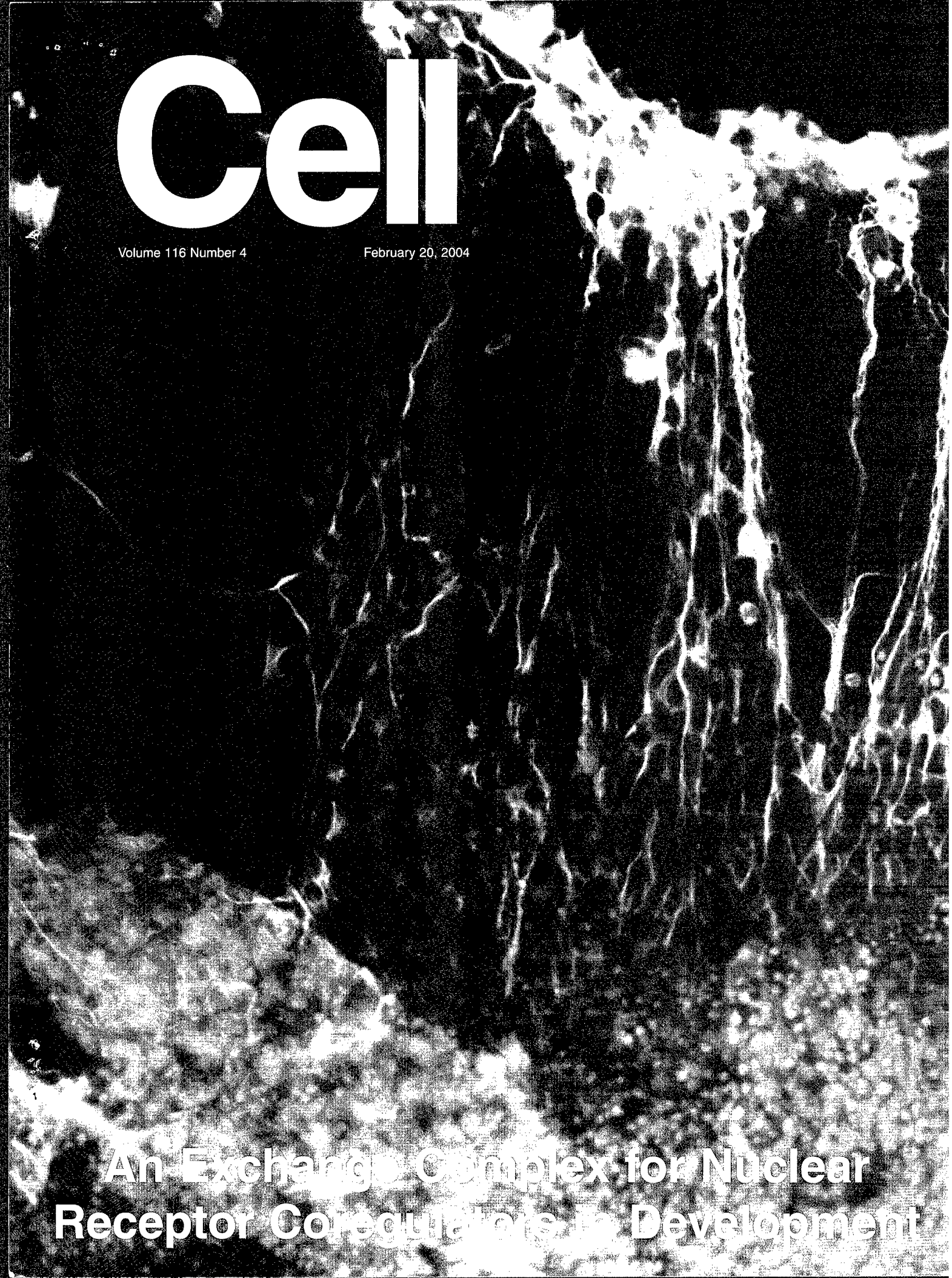

An Exchange Complex for Nuclear Receptor Coregulator Function in Development

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Conversion of Bcl-2 from Protector to Killer by Interaction with Nuclear Orphan Receptor Nur77/TR3

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Summary

The Bcl-2 family proteins are key regulators of apoptosis in human diseases and cancers. Though known to block apoptosis, Bcl-2 promotes cell death through an undefined mechanism. Here, we show that Bcl-2 interacts with orphan nuclear receptor Nur77 (also known as TR3), which is required for cancer cell apoptosis induced by many antineoplastic agents. The interaction is mediated by the N-terminal loop region of Bcl-2 and is required for Nur77 mitochondrial localization and apoptosis. Nur77 binding induces a Bcl-2 conformational change that exposes its BH3 domain, resulting in conversion of Bcl-2 from a protector to a killer. These findings establish the coupling of Nur77 nuclear receptor with the Bcl-2 apoptotic machinery and demonstrate that Bcl-2 can manifest opposing phenotypes, induced by interactions with proteins such as Nur77, suggesting novel strategies for regulating apoptosis in cancer and other diseases.

Introduction

Bcl-2-family proteins are evolutionarily conserved regulators of apoptosis (Adams and Cory, 1998; Gross et al., 1999; Reed, 1998; Vander Heiden and Thompson, 1999). All members possess at least one of the four conserved motifs called Bcl-2 homology (BH) domains. Antiapoptotic members, such as Bcl-2 and Bcl-X_L, contain all four BH domains. Some proapoptotic members, such as Bax and Bak, contain BH1, BH2, and BH3 domains, while others, such as Bad and Bid, share sequence homology only at the BH3 domain. The Bcl-2 family proteins primarily act at mitochondria to regulate apoptosis, possibly by forming channels in mitochondrial membranes (Green and Reed, 1998).

One curious and as yet unexplained aspect of some Bcl-2-family proteins is that their phenotypes can be reversed in some cellular contexts. Overexpression of Bcl-2 or Bcl-X_L in some cells promotes rather than prevents apoptosis, whereas Bax and Bak prevent apoptosis under some circumstances (Chen et al., 1996; Fannjiang et al., 2003; Grandgirard et al., 1998; Lewis et al., 1999; Subramanian and Chinnadurai, 2003; Uhlmann et al., 1998). *Drosophila* Bcl-2 homologs exhibit either pro- or antiapoptotic activity (Colussi et al., 2000; Igaki et al., 2000). Similarly, mutants of the Bcl-2-homolog, Ced-9, appear to promote rather than prevent programmed cell death in *C. elegans* (Xue and Horvitz, 1997). Given that the *C. elegans* genome contains no Bax-homologs, Ced-9 may perform the functions of both Bcl-2 and Bax by adopting different conformations to exert opposing effects on cell life and death.

Nur77 (TR3 or NGFI-B), an orphan member of the steroid/thyroid/retinoid nuclear receptor superfamily (Kastner et al., 1995; Mangelsdorf and Evans, 1995; Zhang, 2002), plays roles in regulating growth and apoptosis (Winoto and Littman, 2002; Zamzami and Kroemer, 2001; Zhang, 2002). Nur77 expression is rapidly induced during apoptosis in immature thymocytes and T cell hybridomas (Liu et al., 1994; Woronicz et al., 1994), and cancer cells of lung (Li et al., 1998; Kolluri et al., 2003), prostate (Li et al., 2000; Uemura and Chang, 1998), ovary (Holmes et al., 2002, 2003), colon (Wilson et al., 2003), and stomach (Liu et al., 2002; Wu et al., 2002). High levels of Nor1, a Nur77-family member, are associated with favorable responses to several chemotherapeutic agents in patients with diffuse large B-cell lymphoma (Shipp et al., 2002).

Recently, we discovered a paradigm in cellular apoptosis (Li et al., 2000), wherein Nur77 translocates from the nucleus to the cytoplasm, targeting to mitochondria and inducing cyt *c* release. Nur77 mitochondrial-targeting occurs during apoptosis of different types of cancer cells (Holmes et al., 2003; Kolluri et al., 2003; Liu et al., 2002; Wilson et al., 2003; Wu et al., 2002). Sindbis virus-induced apoptosis also involves Nur77 translocation to mitochondria (Lee et al., 2002). How Nur77 targets mitochondria and induces apoptosis however has been unclear.

In this study, we investigated the mechanism by which Nur77 targets mitochondria and induces apoptosis. Our results demonstrate that Nur77 interacts with Bcl-2 through its ligand binding domain (LBD) and that the interaction is required for Nur77 mitochondrial targeting and Nur77-dependent apoptosis. Interestingly, Nur77 binds to the Bcl-2 N-terminal loop region, located between its BH4 and BH3 domains, resulting in a conformational change in Bcl-2, which converts it from a protector to a killer protein.

Results

Nur77 Interacts with Bcl-2

We investigated whether Nur77 targets to mitochondria by binding Bcl-2. In vitro protein binding assays showed that similar proportions of ^{35}S-labeled Bcl-2 or RXRα, a known Nur77 heterodimerization partner, were selectively pulled-down by GST-Nur77 but not by GST (Figure 1A). Conversely, ^{35}S-labeled Nur77 and Bax, a known heterodimerization partner of Bcl-2, bound equally to GST-Bcl-2 but not to GST (Figure 1A). In mammalian two-hybrid studies, Bcl-2/ΔTM, a Bcl-2 mutant lacking its C-terminal transmembrane domain, strongly inter-

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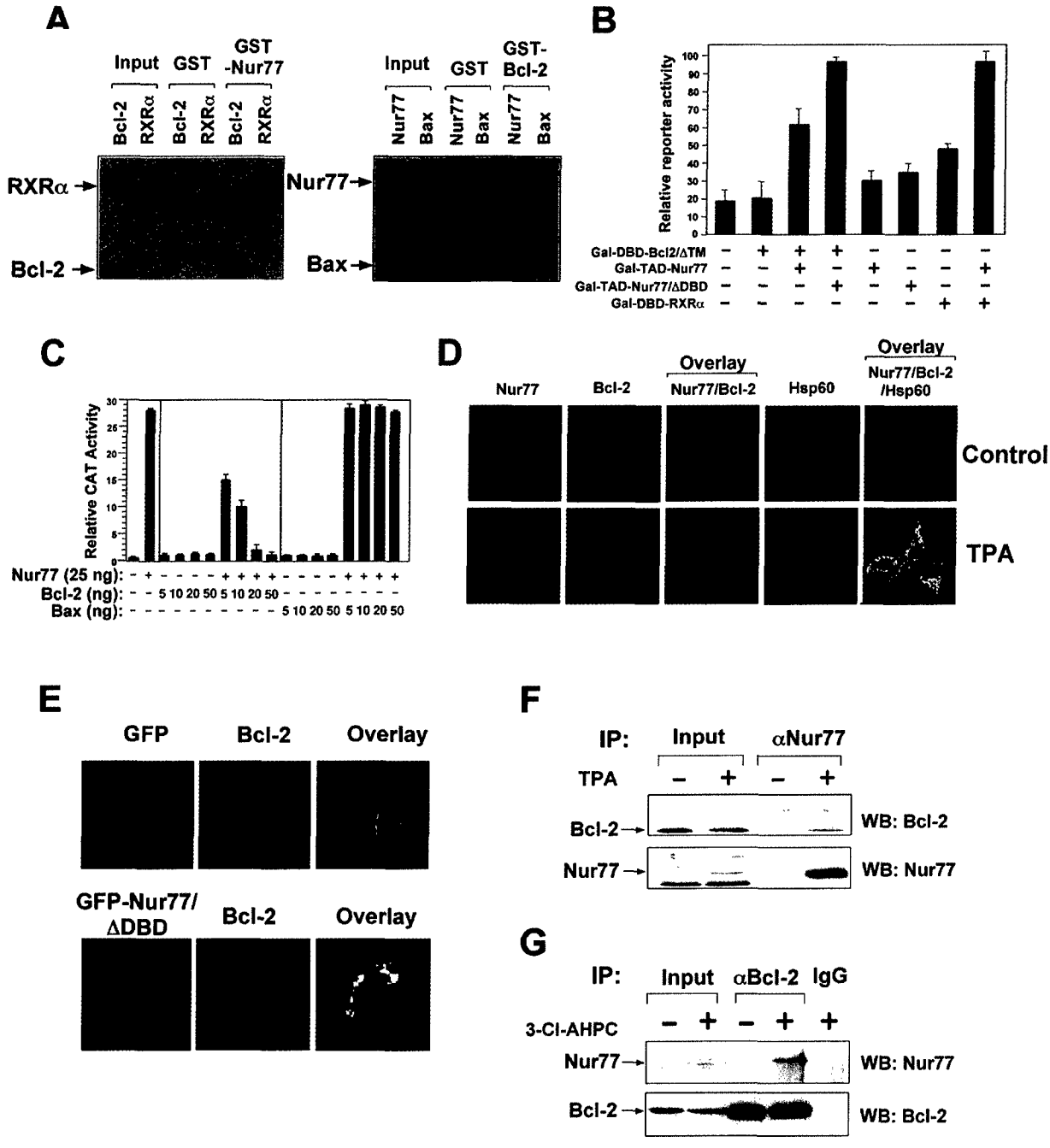

Figure 1. Interaction of Nur77 with Bcl-2

(A) GST-pull down. GST-Nur77, GST-Bcl-2, or GST control protein immobilized on glutathione-Sepharose was incubated with in vitro synthesized ^{35}S-labeled Bcl-2, RXRα, Nur77, or Bax as indicated. Bound proteins were analyzed by SDS-PAGE autoradiography. Input represents 5% of protein used in the pull down assays

(B) Mammalian two-hybrid assay. Gal4 reporter gene $(Gal4)_2$-*tk*-Luc) was transfected into CV-1 cells with the Bcl-2/ΔTM (TM was deleted to prevent Bcl-2 membrane association) or RXRα fused with the Gal-DBD alone or with the Nur77 or Nur77/ΔDBD fused with the Gal-transactivation domain (TAD). Reporter gene activity was determined 48 hr later.

(C) Inhibition of Nur77-dependent transactivation by Bcl-2. CV-1 cells were transfected with the NurRE-*tk*-CAT reporter (Li et al., 2000) with or without Nur77 expression vector together with or without the Bcl-2 or Bax. CAT activity was then determined. The bars in (B) and (C) are means ± SD from three and six experiments, respectively.

(D) Colocalization of endogenous Nur77 and transfected Bcl-2. LNCaP cells were transfected with Bcl-2, treated with or without TPA (100 ng/ml) for 3 hr, then immunostained with polyclonal rabbit anti-Bcl-2, mouse monoclonal anti-Nur77, or anti-Hsp60 antibody. Nur77, Bcl-2, and Hsp60 were visualized using confocal microscopy and images were overlaid (overlay). Approximately 80% of TPA-treated cells demonstrated colocalization.

(E) Colocalization of transfected GFP-Nur77/ΔDBD and Bcl-2. GFP–Nur77/ΔDBD (3 μg) and Bcl-2 (1 μg) were cotransfected into LNCaP cells. After 20 hr, cells were immunostained with anti-Bcl-2 antibody. GFP–fusion and Bcl-2 were visualized as in (D). For control, distribution of transfected GFP empty vector is shown. Approximately 30% of transfected cells exhibited colocalization shown.

(F) In vivo Co-IP assay in LNCaP cells. Lysates from LNCaP cells treated with or without TPA for 3 hr were incubated with mouse monoclonal anti-Nur77 antibody (Abgent, San Diego, CA). For immunoblotting of immunoprecipitates, anti-Bcl-2 or rabbit polyclonal anti-Nur77 antibody (Active Motif, Carlsbad, CA) were used.

(G) In vivo Co-IP in H460 cells. Lysates from H460 cells treated with or without 3-Cl-AHPC (10^{-6} M) for 3 hr were incubated with anti-Bcl-2 antibody. Immunoblotting of immunoprecipitates was conducted as in (F).

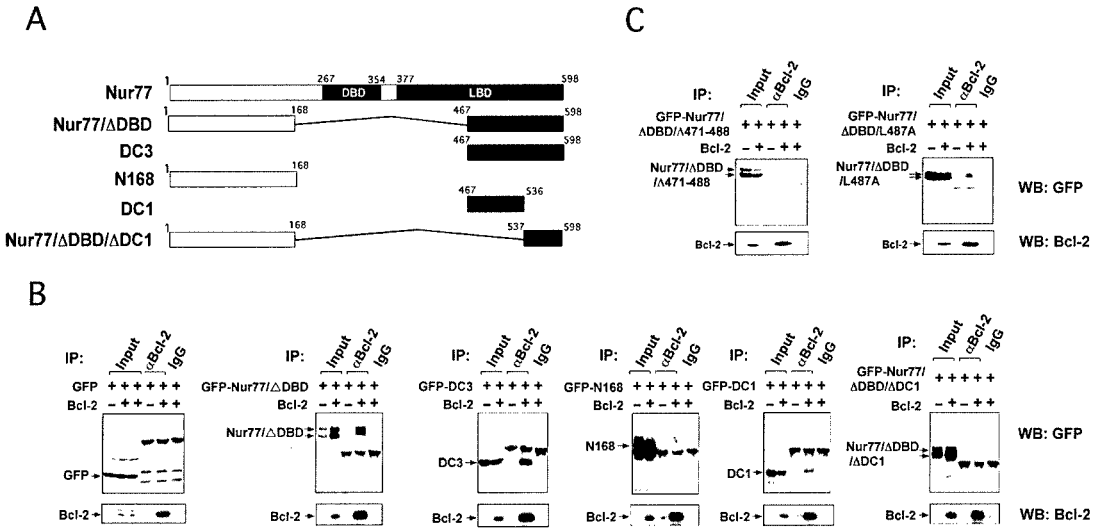

Figure 2. Ligand binding Domain of Nur77 Interacts with Bcl-2

(A) Schematic representation of Nur77 mutants. The Nur77 DBD and LBD are indicated.

(B-C) In vivo Co-IP of Nur77 mutants and Bcl-2. The indicated Nur77 mutant fused with GFP or the empty GFP vector (6 μg) was cotransfected with the empty vector (pRC/CMV) or Bcl-2 expression vector (2 μg) into HEK293T cells. Lysates were immunoprecipitated by using either polyclonal rabbit anti-Bcl-2 antibody (against whole Bcl-2 protein) or control IgG. Cell lysates and immunoprecipitates were examined by immunoblotting using anti-GFP antibody. The same membranes were also blotted with anti-Bcl-2 antibody to determine IP specificity and efficiency. Input represents 5% of cell lysates used in the Co-IP assays.

acted with Nur77 or Nur77/ΔDBD, a Nur77 mutant lacking its DNA binding domain (DBD), comparable to the interaction of Nur77 with RXRα (Figure 1B). Bcl-2 but not Bax potently inhibited Nur77 transactivation (Figure 1C), again suggesting an interaction between Nur77 and Bcl-2.

We next determined whether endogenous Nur77 and transfected Bcl-2 colocalized in cells. The phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) induces the expression of endogenous Nur77 and its mitochondrial localization in LNCaP prostate cancer cells (Li et al., 2000). In TPA-stimulated cells, the distribution patterns of TPA-induced endogenous Nur77 and transfected Bcl-2 overlapped extensively in the cytoplasm and colocalized with Hsp60, a mitochondria-specific protein (Figure 1D). Transfected Nur77/ΔDBD and Bcl-2 also colocalized in cells. The green fluorescent protein (GFP) tagged Nur77/ΔDBD, which constitutively resides on mitochondria in LNCaP cells (Li et al., 2000), displayed a distribution pattern that overlapped extensively with coexpressed Bcl-2, while control GFP protein distributed diffusely in cells (Figure 1E).

The interaction between Nur77 and Bcl-2 was further confirmed by coimmunoprecipitation (Co-IP) assays. As shown in Figure 1F, Bcl-2 was specifically coimmunoprecipitated by anti-Nur77 antibody in TPA-treated cells but not in nontreated cells. Co-IP using lysates from H460 lung cancer cells treated with AHPN analog 3-Cl-AHPC, which potently induces Nur77 expression, mitochondrial targeting and apoptosis (Kolluri et al., 2003), also demonstrated a strong interaction between endogenous Nur77 and Bcl-2 (Figure 1G).

The Nur77 LBD Is Required for Binding Bcl-2

To identify the Nur77 domain responsible for interaction with Bcl-2, we constructed several Nur77 mutants (Figure 2A) as GFP fusions. When GFP-Nur77/ΔDBD and Bcl-2 were cotransfected into HEK293T cells, a significant amount of GFP-Nur77/ΔDBD was coprecipitated with Bcl-2 by anti-Bcl-2 antibody but not by control IgG (Figure 2B). This Co-IP was specific because Bcl-2 coexpression was required and the GFP control protein did not interact with Bcl-2. Analysis of other Nur77 mutants revealed that the C-terminal domain (DC3), but not the N-terminal domain (N168), of Nur77/ΔDBD was responsible for binding Bcl-2. The C-terminal fragment DC1 (467–536 aa) strongly interacted with Bcl-2, while its deletion from Nur77/ΔDBD (Nur77/ΔDBD/ΔDC1) largely abolished the interaction. Furthermore, deletion of a putative amphipathic α-helix (471–488 aa) from Nur77/ΔDBD (Nur77/ΔDBD/Δ471–488) or mutation of Leu487 in the region to Ala (Nur77/ΔDBD/L487A) significantly impaired the interaction between Nur77/ΔDBD and Bcl-2 (Figure 2C). Thus, the DC1 region in the Nur77 LBD is crucial for Bcl-2 interaction.

The Bcl-2 Loop Region, but Not Its Hydrophobic Groove, Is Responsible for Nur77 Binding

Bcl-2 and Bcl-X_L have hydrophobic crevices on their surfaces that bind the BH3 domains of other family members (Sattler et al., 1997). To examine whether Nur77 binds to the Bcl-2 hydrophobic groove, we analyzed the interaction of Nur77/ΔDBD with several deletion or point mutants of Bcl-2 (Figure 3A), which are defective in forming the hydrophobic groove (Petros et al., 2001; Sattler et al., 1997). Deletion (data not shown) or point mutations (Y108K, L137A, G145A, or R146Q) in Bcl-2 abolished or reduced the interaction with Bax (Figure 3B). In contrast, these mutants retained the ability to bind Nur77/ΔDBD (Figure 3C). We also analyzed whether Bax or Bcl-Gs, a BH3-only Bcl-2-family protein (Guo et al., 2001), could compete with Nur77 for binding Bcl-2. Our results

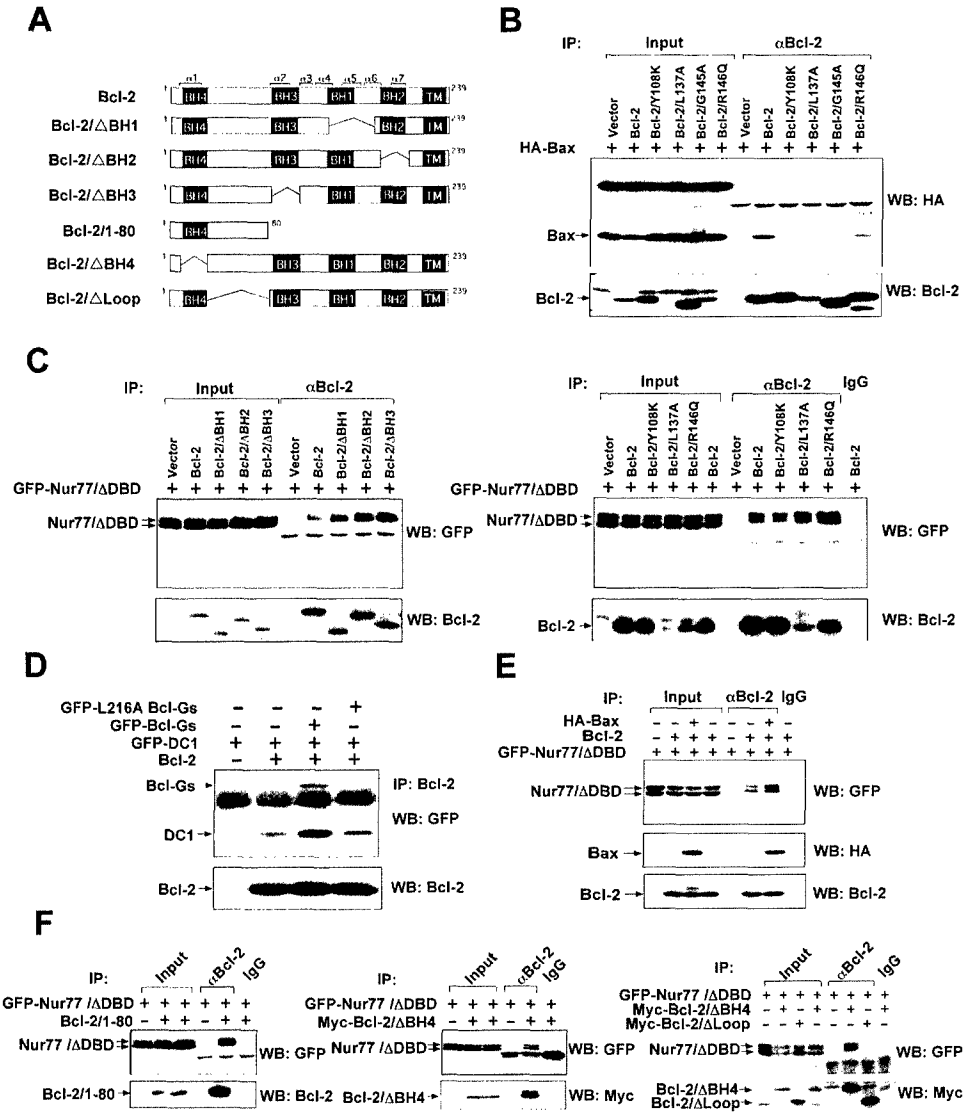

Figure 3. The Bcl-2 Loop Region Interacts with Nur77

(A) Schematic representation of Bcl-2 and its mutants. BH and loop domains and α-helical regions are indicated.

(B) Mutations in the Bcl-2 hydrophobic groove abolish Bcl-2 interaction with Bax.

(C) The Bcl-2 hydrophobic groove is not required for Bcl-2 binding to Nur77/ΔDBD. Co-IP assays (B and C) were performed as in Figure 2B using lysates from HEK293T cells transfected with GFP-Nur77/ΔDBD and the empty vector or the indicated Bcl-2 plasmid. Immunoprecipitates and lysates were examined by immunoblotting using the indicated antibodies.

(D) BH3-only protein Bcl-Gs does not compete with DC1 for binding to Bcl-2. GFP-DC1 (4 μg) was expressed in HEK293T cells with or without Bcl-2 (2 μg) in the presence or absence of GFP-Bcl-Gs or GFP-Bcl-Gs/L216E (4 μg). Lysates were immunoprecipitated by anti-Bcl-2 antibody, followed by immunoblotting with anti-GFP or anti-Bcl-2 antibody.

(E) Bax does not compete with Nur77/ΔDBD for binding to Bcl-2. GFP-Nur77/ΔDBD (6 μg) was expressed in HEK293T cells with or without Bcl-2 (2 μg) in the presence or absence of Bax (2 μg). Lysates were immunoprecipitated by anti-Bcl-2 antibody. Immunoprecipitates and lysates were examined by immunoblotting using anti-GFP, anti-Bcl-2, or anti-HA antibody.

(F) The Bcl-2 N-terminal loop region is essential for Nur77/Bcl-2 interaction. GFP-Nur77/ΔDBD was cotransfected with the indicated Bcl-2 mutant plasmid into HEK293T cells. Lysates were immunoprecipitated by anti-myc or Bcl-2 antibody as indicated. Immunoprecipitates were analyzed by immunoblotting with anti-GFP, anti-Bcl-2, or anti-myc antibody.

showed that neither Bcl-Gs (Figure 3D) nor Bax (Figure 3E) interfered with DC1 or Nur77/ΔDBD binding to Bcl-2. Rather, these proteins consistently enhanced their interaction with Bcl-2. Thus, binding of Bcl-2 to Nur77 is distinct for its binding to Bcl-Gs and Bax and does not require the BH3 binding hydrophobic groove in Bcl-2.

The above observations suggested that the N-terminal portion of Bcl-2 was responsible for binding Nur77. Indeed, the first 80 amino acid residues of Bcl-2 (Bcl-2/1-80), like the full-length Bcl-2, strongly interacted with Nur77/ΔDBD (Figure 3F). The Bcl-2/1-80 encompasses the BH4 domain and an unstructured loop domain. To determine whether the BH4 domain or the loop region was responsible for binding to Nur77/ΔDBD, we investigated Nur77/ΔDBD interaction with Bcl-2 mutants lacking the BH4 domain (Bcl-2/ΔBH4) or the loop region

(Bcl-2/ΔLoop) (Figure 3F). Co-IP assays demonstrated that the Bcl-2/ΔBH4 retained the ability to interact with Nur77/ΔDBD, whereas Bcl-2/ΔLoop did not (Figure 3F). Thus, the loop region of Bcl-2 is required for binding to Nur77.

Interaction with Bcl-2 Mediates Nur77 Mitochondrial Targeting

To determine whether interaction with Bcl-2 mediated Nur77 mitochondrial targeting, we transfected GFP-Nur77/ΔDBD alone or with Bcl-2 into HEK293T cells, which lack detectable levels of endogenous Bcl-2. GFP-Nur77/ΔDBD was diffusely distributed in the cytosol (Figure 4A), indicating that it failed to target mitochondria. However, when coexpressed with Bcl-2, GFP-Nur77/ΔDBD displayed a distribution pattern overlapping with mitochondrial Hsp60. Thus, Bcl-2 expression conferred mitochondrial targeting to Nur77/ΔDBD. The colocalization of Nur77/ΔDBD and Hsp60 was dependent on Nur77/ΔDBD binding to Bcl-2, because mutants of GFP-Nur77/ΔDBD (Nur77/ΔDBD/L487A and Nur77/ΔDBD/Δ471–488) that failed to bind Bcl-2 (Figure 2) did not colocalize with mitochondria.

The role of Bcl-2 in mitochondrial targeting of Nur77 was further studied by examining the accumulation of Bcl-2 binding Nur77/ΔDBD and nonbinding Nur77/ΔDBD/Δ471–488 in mitochondria-enriched heavy membrane (HM) fractions of HEK293T cells (Figure 4B). HM preparation purity was established by assessing levels of mitochondrial Hsp60, nuclear protein PARP, and cytosolic/nuclear protein Jun N-terminal kinase (JNK). Nur77/ΔDBD accumulated in the HM fraction when Bcl-2 was coexpressed, whereas Nur77/ΔDBD/Δ471-488 did not accumulate irrespective of Bcl-2 coexpression.

To complement these gene transfection experiments, small interfering (si)RNA was used to determine whether suppressing endogenous Bcl-2 expression affected Nur77 mitochondrial targeting. In MGC80-3 gastric cancer cells, in which Nur77 was reported to target mitochondria in response to specific apoptotic stimuli (Liu et al., 2002), Bcl-2 expression was almost completely inhibited by Bcl-2-specific siRNA but not by GFP siRNA control (Figure 4C). Both confocal microscopy (Figure 4D) and immunoblotting of HM fractions (Figure 4E) revealed that endogenous Nur77 targeted mitochondria in MGC80-3 cells treated with 3-Cl-AHPC. However, Bcl-2 siRNA, but not control GFP siRNA, largely abolished mitochondrial targeting of Nur77. Similarly, inhibition of endogenous Bcl-2 expression using Bcl-2 antisense oligonucleotides impaired Nur77 mitochondrial targeting in H460 lung cancer cells (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/116/4/527/DC1).

We next studied whether the Bcl-2 loop region could act in a dominant-negative fashion to inhibit Nur77 mitochondrial targeting. In LNCaP cells transfected with GFP-Bcl-2/1-90, a Bcl-2 mutant comprised of the first 90 N-terminal amino acids, TPA-induced Nur77 failed to target mitochondria, displaying a diffuse cytosolic distribution pattern, in contrast to nontransfected cells, which exhibited colocalization of Nur77 and Hsp60 (Figure 4F). Thus, Bcl-2/1-90 inhibits Nur77 mitochondrial targeting, probably by competing with endogenous Bcl-2 for binding to Nur77. Together, these results demonstrate that Bcl-2 acts as a receptor for Nur77 and is responsible for Nur77 mitochondrial targeting.

Nur77 Interaction with Bcl-2 Triggers cyt *c* Release and Apoptosis

Next, we determined the requirement of Nur77 interaction with Bcl-2 for Nur77-induced cyt *c* release and apoptosis. Transient expression of Nur77/ΔDBD or Bcl-2 alone did not cause release of cyt *c* from mitochondria in HEK293T cells, as confocal microscopy analysis showed punctate cyt c staining, indicative of mitochondrial cyt *c* (Figure 5A). However, their coexpression resulted in their colocalization and release of cyt *c* from mitochondria (Figure 5A). Cyt *c* release required mitochondrial localization of Nur77/ΔDBD and Bcl-2, because it did not occur upon coexpression of Nur77/ΔDBD with Bcl-2/ΔTM, a Bcl-2 mutant unable to target mitochondria (Figure 5A). Interestingly, coexpression of Nur77/ΔDBD and Bcl-2/Y108K did not induce cyt *c* release, although they colocalized (Figure 5A). These results suggest that the interaction between Nur77/ΔDBD and Bcl-2 is insufficient for inducing cyt *c* release.

Expression of either Nur77/ΔDBD or Bcl-2 alone did not induce apoptosis, as revealed by the absence of nuclear fragmentation and chromatin condensation in HEK293T cells (Figure 5B). However, when coexpressed, Bcl-2 and Nur77/ΔDBD induced striking apoptosis. The proapoptotic effect of Bcl-2 was specific to Nur77, because Bax-induced apoptosis was effectively prevented by Bcl-2 coexpression (see below). Thus, Bcl-2 promotes apoptosis when coexpressed with Nur77 but suppresses apoptosis when coexpressed with Bax.

We next examined the role of endogenous Bcl-2 on Nur77-dependent apoptosis in MGC80-3 cells. Treatment of control GFP-siRNA-transfected cells with 3-Cl-AHPC resulted in apoptosis (Figures 5C and 5D). However, transfection of Bcl-2 siRNA suppressed 3-Cl-AHPC-induced apoptosis by about 60%. Similar results were obtained in H460 cells (Supplemental Figure S1 available on *Cell* website). Moreover, expression of Bcl-2/1-90 protein also suppressed Nur77-dependent apoptosis induced by TPA and 3-Cl-AHPC in LNCaP cells (Figure 5E). Thus, Bcl-2 can manifest a proapoptotic phenotype in settings where Nur77 is expressed and targets to mitochondria.

To extend the above findings to primary cells, we performed experiments using primary cultures of peripheral blood lymphocytes (PBLs). Freshly isolated PBLs were transfected with GFP-Nur77, then treated with TPA plus calcium ionophore ionomycin, which induce Nur77-dependent apoptosis of T-lymphocytes (Woronicz et al., 1994). The treatment caused translocation of GFP-Nur77 from the nucleus to the cytoplasm, colocalizing with cotransfected DsRed2-Mito, a red fluorescent protein (RFP) fused with a mitochondria-targeting sequence (Figure 6A). Subcellular fractionation revealed that the treatment induced accumulation of endogenous Nur77 in HM fractions (Figure 6B). Interestingly, this treatment also altered the migration of Nur77 protein, suggesting a possible posttranslational modification. Thus, both transfected and endogenous Nur77 targets mitochondria in primary lymphocytes.

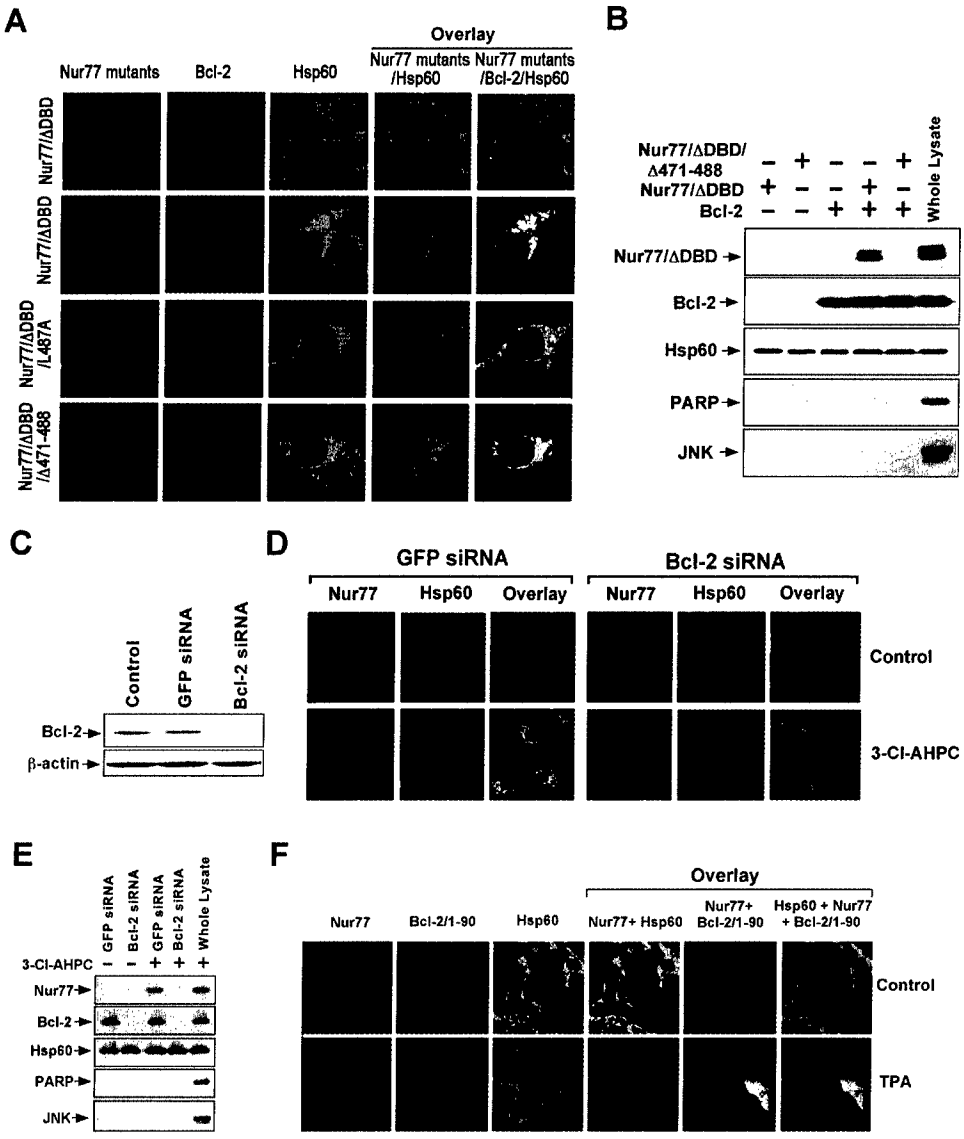

Figure 4. Nur77/Bcl-2 Interaction Mediates Nur77 Mitochondrial Localization

(A) Bcl-2 expression promotes Nur77/ΔDBD mitochondrial localization. The indicated GFP-Nur77 mutant (3 μg) and Bcl-2 (1 μg) were expressed in HEK293T cells alone or together. Cells were immunostained with anti-Bcl-2 or anti-Hsp60 antibody. Bcl-2, Nur77/ΔDBD, its mutants, and mitochondria (Hsp60) were visualized using confocal microscopy and the images were overlaid (overlay). Approximately 30% of cells showed Nur77/ΔDBD colocalization with Bcl-2 and Hsp60, while less than 5% of cells transfected with Nur77/ΔDBD mutants were similarly colocalized. In the absence of Bcl-2, Nur77/ΔDBD did not colocalize with Hsp60.

(B) Immunoblotting analysis of the effect of Bcl-2 expression. GFP-Nur77/ΔDBD or GFP-Nur77/ΔDBD/Δ471-488 (6 μg) and Bcl-2 (2 μg) were transfected into HEK293T cells alone or together. HM fractions were prepared and analyzed for accumulation of Nur77/ΔDBD in mitochondria by immunoblotting using anti-GFP antibody. The same membrane was also blotted with anti-Bcl-2, anti-Hsp60, anti-PARP, or anti-JNK antibody. Whole lysate was prepared from cells transfected with Nur77/ΔDBD and Bcl-2.

(C) Inhibition of Bcl-2 expression by Bcl-2 siRNA. MGC80-3 cells were transfected with Bcl-2 siRNA SMARTpool or control GFP siRNA or left alone. After 48 hr, lysates were prepared and assayed by immunoblotting using anti-Bcl-2 and anti-β-actin antibodies.

(D–E) Inhibition of endogenous Bcl-2 expression abrogates 3-Cl-AHPC-induced Nur77 mitochondrial targeting. MGC80-3 cells transfected with siRNA as described in (C) were treated with 3-Cl-AHPC (10^{-6} M) for 5 hr. Cells were immunostained with anti-Nur77 and anti-Hsp60 antibodies for confocal microscopy analysis (D) or subjected to HM fractionation and analysis (E) as described in (B).

(F) Bcl-2/1-90 inhibits Nur77 mitochondrial targeting. LNCaP cells were transfected with GFP-Bcl-2/1-90 (4 μg). After 24 hr, cells were treated with TPA (100 ng/ml) for 3 hr and immunostained with anti-Nur77 and anti-Hsp60 antibodies, followed by confocal microscopy analysis. Approximately 77% of transfected cells showed the effect presented.

We also studied the role of Bcl-2 in Nur77-dependent apoptosis in PBLs. Treatment with TPA/ionomycin induced extensive apoptosis of PBLs, which was partially inhibited by Bcl-2 antisense oligonucleotides or Nur77 siRNA (Figure 6C). In addition, GFP-Nur77/ΔDBD also colocalized extensively with DsRed2-Mito (Figure 6D) and potently induced PBL apoptosis (Figure 6E), which was almost completely suppressed by Bcl-2 antisense

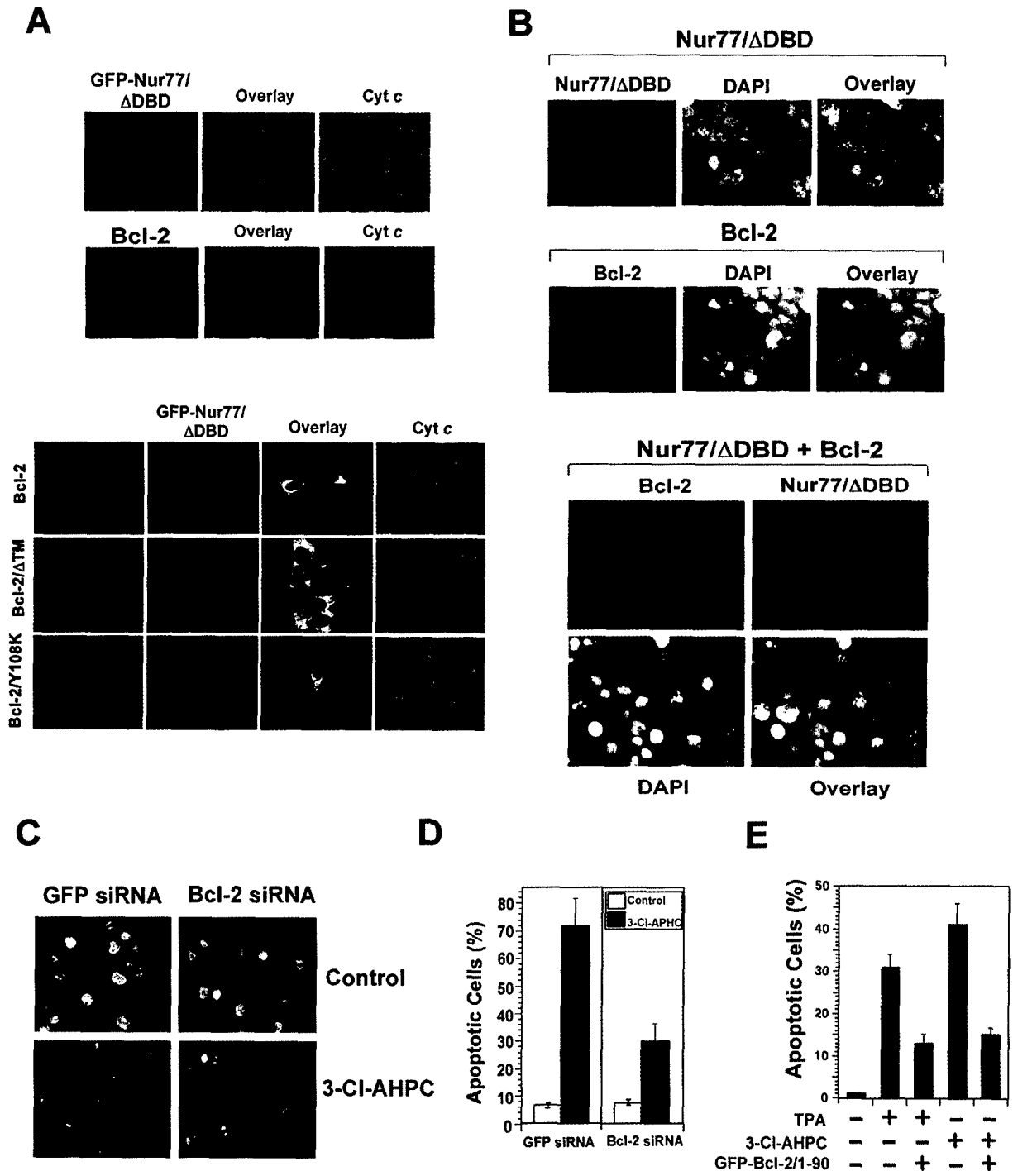

Figure 5. Interaction of Nur77 with Bcl-2 Results in cyt *c* Release and Apoptosis

(A) Induction of cyt *c* release by coexpression of Bcl-2 and Nur77/ΔDBD. GFP-Nur77/ΔDBD (6 μg) and Bcl-2 (2 μg) were expressed in HEK293T cells alone and together. GFP-Nur77/ΔDBD was also coexpressed with Bcl-2/ΔTM or Bcl-2/Y108K (2 μg). Cells were immunostained with anti-Bcl-2 or anti-cyt c antibody. Nur77/ΔDBD, Bcl-2 and cyt *c* were visualized using confocal microscopy, and images for Nur77/ΔDBD and Bcl-2 were overlaid (overlay). Approximately 75% of the Nur77/ΔDBD and Bcl-2 colocalized cells displayed various levels of diffuse cyt *c* staining.

(B) Induction of apoptosis by coexpression of Bcl-2 and Nur77/ΔDBD. Bcl-2 and GFP-Nur77/ΔDBD were expressed alone or together in HEK293T cells. After 36 hr, cells were stained by anti-Bcl-2 antibody, followed by TRITC-conjugated secondary antibody (Sigma) and the nucleus was stained by DAPI. Expression of Bcl-2 and GFP-Nur77/ΔDBD, as well as nuclear morphology, were visualized by fluorescence microscopy, and the three images were overlaid. Arrows indicate cells expressing Bcl-2 and GFP-Nur77/ΔDBD. One of four similar experiments is shown.

(C–D) Inhibition of endogenous Bcl-2 expression suppresses 3-Cl-AHPC-induced apoptosis. MGC80-3 cells were transfected with Bcl-2 siRNA SMARTpool or control GFP siRNA. After 36 hr, cells were treated with 3-Cl-AHPC (10^{-6} M) for 48 hr. Apoptosis was determined by DAPI staining as shown in (C) and scored by examining 300 cells for nuclear fragmentation and/or chromatin condensation (D).

(E) Bcl-2/1-90 inhibits TPA and 3-Cl-AHPC-induced apoptosis. LNCaP cells were transfected with GFP or GFP-Bcl-2/1-90. After 24 hr, cells were treated with TPA (100 ng/ml) or 3-Cl-AHPC (10^{-6} M) for 24 hr. Apoptosis was studied as in D.

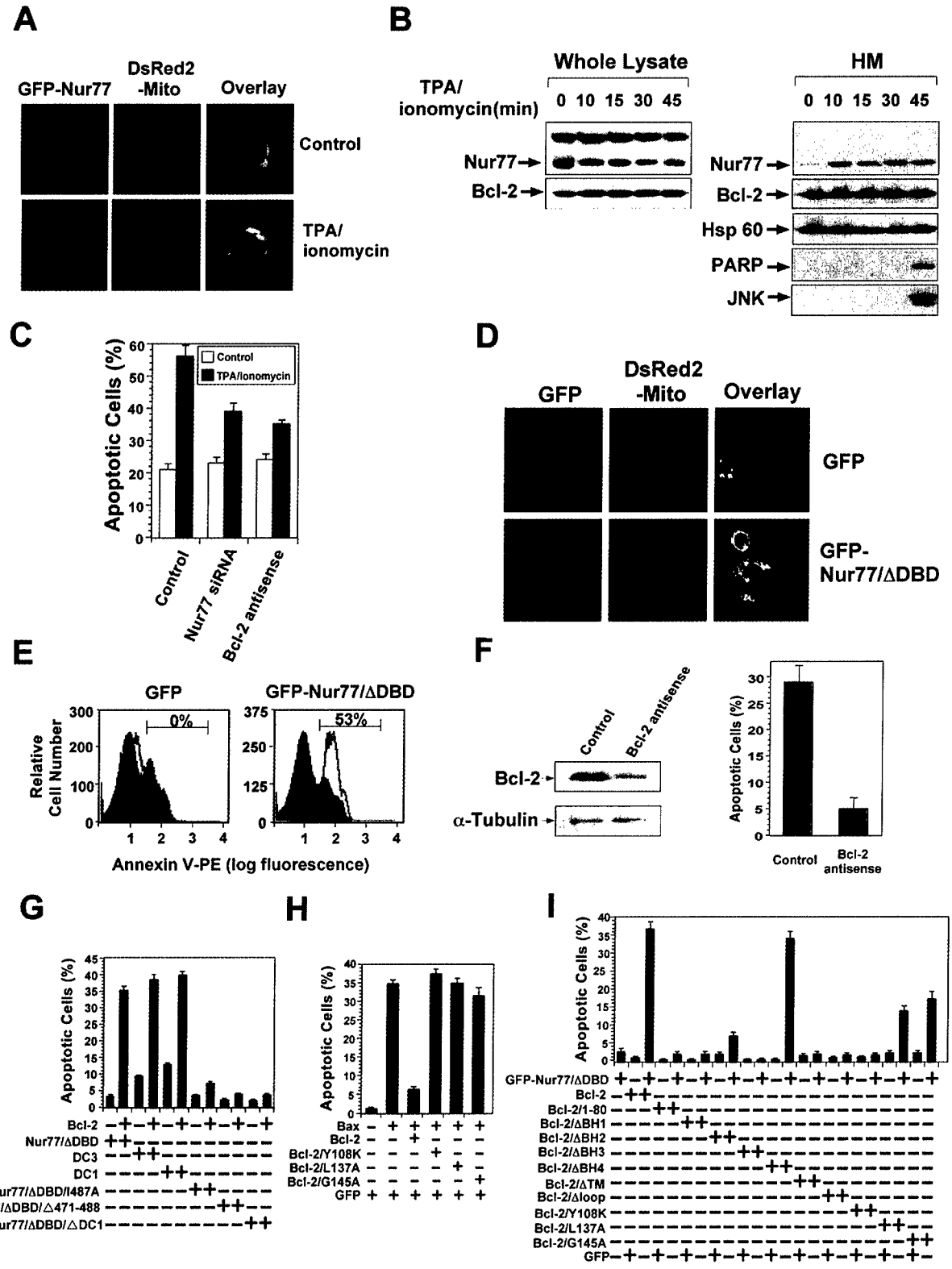

Figure 6. Nur77 Mitochondrial Targeting in Human PBLs and Apoptotic Effects of Nur77 and Bcl-2 Mutants

(A) Mitochondrial targeting of Nur77 in PBLs. GFP-Nur77 (1 μg) and pDsRed2-Mito (1 μg) were transfected into freshly isolated human PBLs. The cells were then treated with TPA (10 ng/ml) and ionomycin (0.5 μM) for 30 min after 10 hr of transfection. GFP-Nur77 and mitochondria (pDsRed2-Mito) were visualized using confocal microscopy. Approximately 20% of the cells showed the pattern presented.

(B) Endogenous Nur77 accumulates in the PBL HM fraction. PBLs were treated with TPA and ionomycin as in (A) for the indicated times and HM fractions were isolated. Total cell lysates and HM fractions were subjected to immunoblotting as described in Figure 4B.

(C) Nur77 and Bcl-2 are required for apoptosis in PBLs. PBLs were transfected with control GFP siRNA, Nur77 siRNA, or Bcl-2 antisense oligonucleotides (2 μg). After 40 hr, cells were treated with TPA and ionomycin for 7 hr and apoptotic cells (Annexin-V positive) were determined by flow cytometry. Bars represent average ± means from two experiments.

oligonucleotides (Figure 6F). Thus, endogenous Bcl-2 contributes to Nur77-dependent apoptosis in primary lymphocytes.

Analysis of Bcl-2 Domain Required for Apoptosis Induction by Nur77

To characterize the proapoptotic mechanism of Bcl-2 in Nur77-induced apoptosis, various Nur77 and Bcl-2 mutants were coexpressed in HEK293T cells. Similar to Nur77/ΔDBD, coexpression of either DC3 or DC1 with Bcl-2 strongly induced apoptosis (Figure 6G). Thus, the minimal C-terminal domain of Nur77, capable of binding Bcl-2, was sufficient to induce apoptosis when coexpressed with Bcl-2. The requirement of Nur77 interaction with Bcl-2 for apoptosis was further illustrated by the failure of Nur77 mutants (Nur77/ΔDBD/ΔDC1, Nur77/ΔDBD/Δ471-488, and Nur77/ΔDBD/L487A) that failed to bind Bcl-2 (Figure 2) to induce apoptosis when coexpressed with Bcl-2 (Figure 6G).

Bcl-2 effectively suppressed apoptosis induced by Bax expression in HEK293T cells (Figure 6H). Bcl-2 mutations (Y108K, L137A, G145A) that impaired its interaction with Bax (Figure 3B) abolished its inhibitory effect on Bax-induced apoptosis (Figure 6H), consistent with previous observations that the Bcl-2 hydrophobic cleft is essential for its antiapoptotic effect.

We then performed experiments to delineate the structure-function relationships for the proapoptotic effect of Bcl-2 in Nur77-induced apoptosis (Figure 6I). Coexpression of Nur77/ΔDBD with Bcl-2/ΔLoop did not induce cell death, consistent with the inability of this Bcl-2 mutant to bind Nur77 (Figure 3). Though capable of binding Nur77/ΔDBD, mutants of Bcl-2 lacking the membrane-anchoring TM domain, the BH1 domain, BH2 domain, or BH3 domain were incapable of inducing apoptosis when coexpressed with Nur77/ΔDBD. Similarly, although binding Nur77, a BH3 domain mutant of Bcl-2 (Y108K) also failed to induce apoptosis when coexpressed with Nur77/ΔDBD. Moreover, mutations of the BH3 binding pocket of Bcl-2, L137A, and G145A, which abrogated the ability of Bcl-2 to suppress Bax-induced apoptosis (Figure 6H), retained the ability to promote apoptosis when coexpressed with Nur77/ΔDBD. Thus, an intact hydrophobic groove in Bcl-2 is required for its antiapoptotic activity but not for its proapoptotic activity, demonstrating a structural distinction between these two opposing phenotypes of Bcl-2.

Bcl-2 Undergoes a Conformational Change upon Nur77 Binding

Bax and Bak undergo conformational changes in association with their conversion from latent to active killer proteins (Griffiths et al., 1999; Nechushtan et al., 1999). We therefore explored whether a conformational change might be involved in converting Bcl-2 function from antiapoptotic to proapoptotic. To this end, we compared the effects of Nur77 on binding of Bcl-2 to various anti-Bcl-2 antibodies that recognize different epitopes. Antibody binding to Bcl-2 was measured by immunofluorescence using flow cytometry or by immunoprecipitation.

First, Bcl-2 was coexpressed with GFP-Nur77/ΔDBD or the control GFP in HEK293T cells, and immunostaining was performed on fixed and permeabilized cells using rabbit polyclonal antibody against the whole Bcl-2 protein (αBcl-2), mouse monoclonal antibody against the Bcl-2 BH3 binding pocket (αBcl-2/BH3-pocket), or polyclonal antibody against the Bcl-2 BH3 domain (αBcl-2/BH3-domain) (Figure 7A). Bcl-2 immunofluorescence was undetectable in control GFP-coexpressing cells stained with the αBcl-2/BH3-domain antibody but dramatically increased in GFP-Nur77/ΔDBD-coexpressing cells, suggesting increased availability of the BH3-domain epitope upon Nur77/ΔDBD coexpression (Figure 7A). In contrast, immunofluorescence obtained by staining with the αBcl-2/BH3-pocket antibody was reduced by coexpression of GFP-Nur77/ΔDBD, suggesting decreased availability of this epitope. Alterations in binding of epitope-specific antibodies to Bcl-2 in response to GFP-Nur77/ΔDBD coexpression were not due to changes in Bcl-2 levels, because GFP-Nur77/ΔDBD coexpression did not alter Bcl-2 immunofluorescence when stained with αBcl-2 antibody. In addition, both immunoblotting analysis (Figure 7A) and BD cytometric bead assays (Supplemental Figure S2 available on *Cell* website) revealed equivalent Bcl-2 levels with GFP or GFP-Nur77/ΔDBD coexpression. Nur77/ΔDBD coexpression also did not modify binding of these epitope-specific antibodies to Bcl-2/ΔLoop (Supplemental Figure S3 available on *Cell* website). The Nur77/ΔDBD-induced change in Bcl-2 conformation was also observed in PBLs (Figure 7B and Supplemental Figure S4 available on *Cell* website).

Second, the effects of Nur77/ΔDBD on Bcl-2 conformation were studied using immunoprecipitation assays. These experiments showed that coexpression of Nur77/ΔDBD reduced binding of Bcl-2 to the αBcl-2/BH3-

(D) Nur77/ΔDBD targets mitochondria in PBLs. pDsRed2-Mito and GFP or GFP-Nur77/ΔDBD (1 μg each) were cotransfected into PBLs. GFP-Nur77/ΔDBD and pDsRed2-Mito were visualized as described in (A). Approximately 30% of the transfected cells showed the pattern presented.
(E) Nur77/ΔDBD induces apoptosis of PBLs. GFP or GFP-Nur77/ΔDBD (2 μg) was transfected into PBLs for 18 hr. The transfected (GFP-positive) cell subpopulation was identified by flow cytometry (Kolluri et al., 1999). The apoptotic cells in the transfected (green histogram) and nontransfected (purple histogram) cells were identified by Annexin-V-PE staining. The numbers represent % of transfected cells showing Annexin-V staining compared to nontransfected cells from the same culture dish.
(F) Bcl-2 is required for Nur77/ΔDBD-induced apoptosis. GFP-Nur77/ΔDBD (1 μg) was cotransfected into PBLs with control oligonucleotides or Bcl-2 antisense oligonucleotides (2 μg). After 48 hr, apoptotic cells were determined as described for (E). Bars represent average ± means from two measurements.
(G) Interaction of Nur77 with Bcl-2 is required for apoptotic effect of Nur77/Bcl-2 coexpression. The indicated Nur77 mutant (6 μg) was transfected with empty or Bcl-2 expression vector (2 μg) into HEK293T cells. After 36 hr, apoptotic cells were determined by DAPI staining.
(H) Effect of Bcl-2 mutations on the apoptotic effect of Bax. HEK293T cells were transfected with the indicated expression vectors and apoptotic cells were determined by DAPI staining.
(I) Effect of Bcl-2 mutations on the apoptotic effect of Nur77/Bcl-2 coexpression. Bcl-2 or a mutant was transfected with GFP or GFP-Nur77/ΔDBD into HEK293T cells. Apoptosis wan then determined by DAPI staining. Bars in (G)–(I) are means ± SD from three independent experiments.

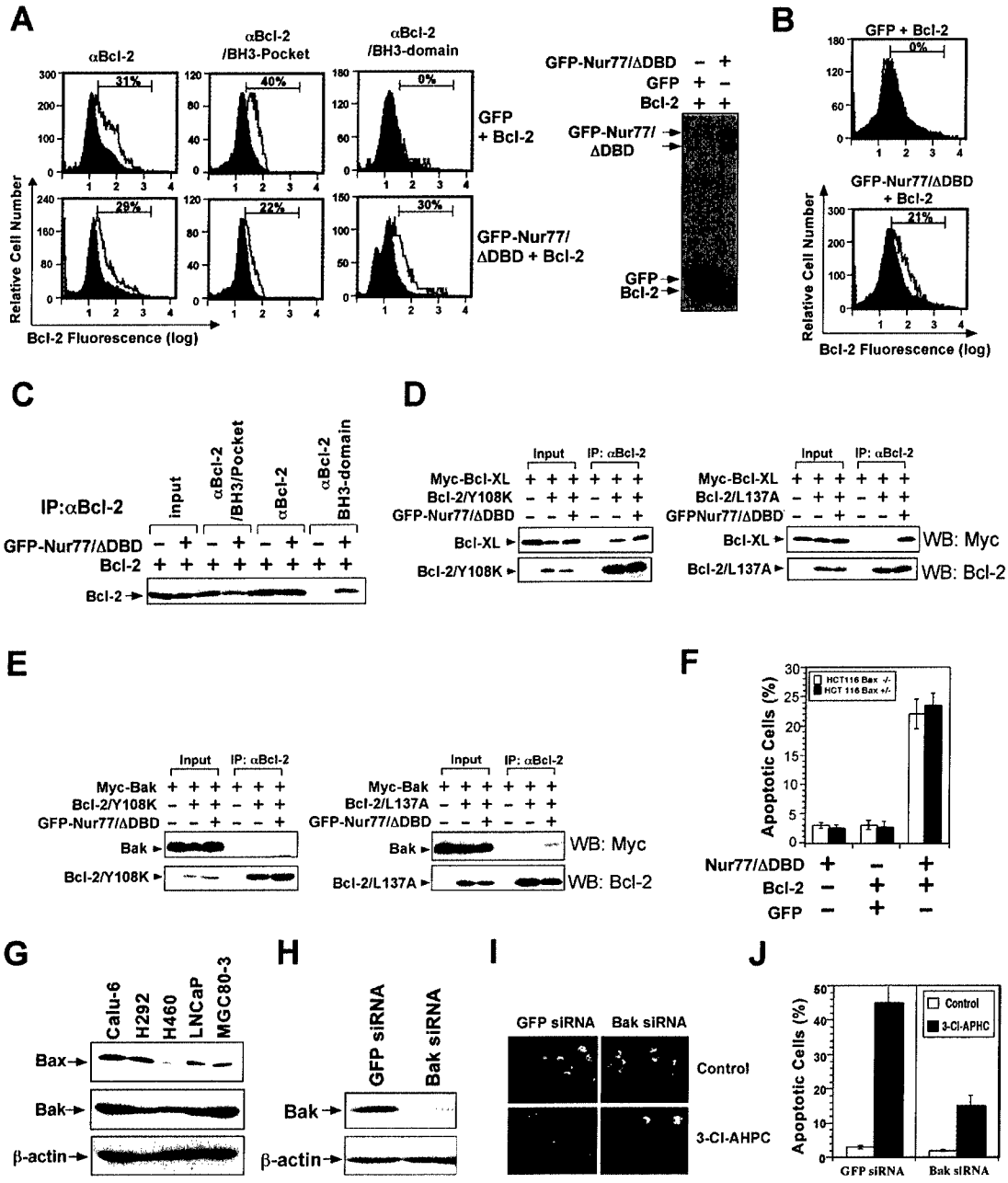

Figure 7. Interaction of Nur77 with Bcl-2 Induces Bcl-2 Conformational Change

(A) Nur77 induces change in Bcl-2 conformation. Bcl-2 (5 μg) was cotransfected into HEK293T cells with GFP or GFP-Nur77/ΔDBD (5 μg) for 14 hr. Portion of the transfected cells were subjected to immunoblotting to confirm similar expression of Bcl-2 in the two samples (right). The remaining cells were divided into three different pools, which were immunostained with three different anti-Bcl-2 antibodies: αBcl-2, αBcl-2/BH3-pocket (BD Transduction Labs), and αBcl-2/BH3 domain (Abgent), followed by SRPD-conjugated secondary antibody (Southern Biotech). Transfected (GFP-positive) cells were identified by flow cytometry. Bcl-2 fluorescence from the transfected cells (green histogram) was compared to that from the nontransfected cells (purple histogram). Similarly fluorescing cells were gated to compare Bcl-2 immunofluorescence after GFP or GFP-Nur77/ΔDBD coexpression. Numbers represent % of transfected cells showing Bcl-2 immunofluorescence compared to the autofluorescence of the nontransfected cells from the same transfection.

(B) Change of Bcl-2 conformation by Nur77/ΔDBD in PBLs. Bcl-2 (1 μg) was cotransfected into PBLs with GFP or GFP-Nur77/ΔDBD (1 μg). A portion of the cells were subjected to immunoblotting (Supplemental Figure S4 available on *Cell* website). The remaining cells were immunostained with αBcl-2/BH3-domain antibody 10 hr after transfection as described in (A).

(C) Nur77/ΔDBD modulates immunoprecipitation of Bcl-2 by epitope-specific anti-Bcl-2 antibodies. HEK293T cells were transfected with the indicated expression vector. After 18 hr, cells lysates were prepared and incubated with the indicated anti-Bcl-2 antibody for immunoprecipitation. Immunoprecipitates were subjected to immunoblotting using anti-Bcl-2 antibody (Santa Cruz).

(D) Alteration of Bcl-2 interaction with Bcl-X_L by Nur77/ΔDBD. The indicated Bcl-2 mutant and Bcl-X_L were coexpressed with or without GFP-Nur77/ΔDBD in HEK293T cells. Lysates were immunoprecipitated by anti-Bcl-2 antibody, and immunoprecipitates examined by immunoblotting using anti-Myc or anti-Bcl-2 antibody.

(E) Alteration of Bcl-2 interaction with Bak by Nur77/ΔDBD. The indicated Bcl-2 mutant and Bak were coexpressed with or without GFP-

pocket antibody, but enhanced binding of Bcl-2 to the αBcl-2/BH3-domain antibody. In contrast, Nur77/ΔDBD did not affect the immunoprecipitation efficiency of the αBcl-2 antibody (Figure 7C). Together, these results demonstrate that Nur77 binding induces a Bcl-2 conformational change that exposes its BH3 domain.

Proapoptotic BH3-only members of the Bcl-2 family induce apoptosis by binding to other Bcl-2 family members through their BH3 domains (Huang and Strasser, 2000; Kelekar and Thompson, 1998). We therefore examined whether Nur77 binding alters the ability of Bcl-2 to bind Bcl-X_L or Bak (Figures 7D-7E). At least when assessed in detergent containing cell lysates by Co-IP, Bcl-2 bound Bcl-X_L and Bak independently of Nur77 (data not shown). To address whether Bcl-2 bound differently to Bcl-X_L and Bak in the presence of Nur77/ΔDBD, two Bcl-2 mutants were analyzed. Bcl-2/L137A, a BH3 binding pocket mutant that retained killing activity in the presence of Nur77/ΔDBD, interacted with Bcl-X_L and Bak only when Nur77/ΔDBD was coexpressed. In contrast, binding of Bcl-X_L and Bak to the Bcl-2/Y108K BH3 domain mutant was unaffected by coexpression of Nur77 (Figures 7D and 7E). Thus, Nur77 binding may result in altered association of Bcl-2 with other Bcl-2 family members. Moreover, the observation that Bcl-2/L137A, but not Bcl-2/Y108K, was capable of killing cells in collaboration with Nur77/ΔDBD (Figure 6I) suggests that exposure of the BH3 domain of Bcl-2 may be responsible for the conversion of Bcl-2 to a proapoptotic molecule.

The above data suggest that Bcl-2, upon Nur77 binding, induces apoptosis through its BH3 domain. BH3-only proteins exert their apoptotic effects through either Bax or Bak. We therefore examined the involvement of Bax and Bak in Bcl-2-dependent apoptosis induced by Nur77. Coexpression of Nur77/ΔDBD and Bcl-2 resulted in a similar degree of apoptosis in HCT116 cells and HCT116 cells lacking Bax (HCT116 $Bax^{-/-}$) (Figure 7F), suggesting that expression of Bax is not crucial. This was also supported by the observation that H460 cells, which underwent extensive apoptosis in response to 3-Cl-AHPC (Supplemental Figure S1 available on *Cell* website), expressed only trace levels of Bax (Figure 7G).

To determine whether Bak, which was highly expressed in H460 cells (Figure 7G), plays a role in Bcl-2-dependent apoptosis induced by Nur77, we examined the effects of suppressing endogenous Bak expression. Significant reductions of Bak protein were observed when H460 cells were transfected with Bak siRNA but not control siRNA (Figure 7H), correlating with significant repression of Nur77-dependent 3-Cl-AHPC-induced apoptosis (Figures 7I–7J). Thus, Bcl-2-mediated apoptosis induced by Nur77 depends on multidomain proapoptotic Bcl-2-family proteins such as Bak.

Discussion

Despite lacking classical mitochondria-targeting sequences, Nur77 translocates from the nucleus to mitochondria, in response to specific cell death stimuli, to trigger cyt *c* release and apoptosis. The results presented here provide evidence that Nur77 targets mitochondria through its interaction with Bcl-2, revealing a crosstalk between Nur77 nuclear receptor and the Bcl-2 signalings. Furthermore, our results demonstrate that the interaction provokes a proapoptotic phenotype of Bcl-2 by inducing a conformational change in Bcl-2 that results in exposure of its BH3 domain. Given that Bcl-2 has been shown to have proapoptotic phenotypes in a variety of contexts, it will be interesting to explore in the future whether Nur77 serves as the mediator of this phenotypic conversion versus other Bcl-2 binding proteins that may await discovery.

Our mutagenesis studies indicate that the loop region located between the BH4 and BH3 domains of Bcl-2 is required for Nur77 binding. Previous studies (Chang et al., 1997) demonstrated that the loop regions of Bcl-2 and Bcl-X_L act as an autoinhibitory domain that reduces the antiapoptotic function of Bcl-2 and Bcl-X_L. Our data suggest that this conserved loop segment found in the vertebrate orthologs of Bcl-2 and Bcl-X_L may also participate in converting the phenotype of Bcl-2 from a protector to a killer of cells. Intriguingly, deletion of the loop region of Bcl-2 blocks paclitaxel-induced apoptosis (Srivastava et al., 1999), thereby suggesting the requirement of the loop region for the apoptotic effect of certain anticancer drugs. Though controversial, paclitaxel may also bind directly to the Bcl-2 loop domain to exert its apoptotic effect (Rodi et al., 1999). For Bcl-X_L, deamidation of residues in its loop region is associated with down regulation of its antiapoptotic activity (Deverman et al., 2002). In another study, insulin receptor substrate (IRS) protein binds to the Bcl-2 loop region, enhancing rather than inhibiting its antiapoptotic function (Ueno et al., 2000). It would be interesting therefore to study whether Nur77 and IRS compete for binding to Bcl-2, exhibiting opposing effects on apoptosis.

The Bcl-2 family members can be divided into two functional subgroups based on whether the BH3 domain is available (Gross et al., 1999). Members with buried BH3 domains are antiapoptotic, while members having an exposed BH3 domain are proapoptotic. Our analysis

Nur77/ΔDBD in HEK293T cells. Lysates were immunoprecipitated by anti-Bcl-2 antibody, and immunoprecipitates examined by immunoblotting using anti-Myc or anti-Bcl-2 antibody.

(F) Absence of Bax does not impair the apoptotic effect of Nur77/ΔDBD. Nur77/ΔDBD and Bcl-2 alone and together were transfected into HCT116 cells (+/−) and HCT116 cells lacking Bax (−/−). After 36 hr, apoptotic cells were determined as in Figure 5D.

(G) Expression of Bax and Bak in cancer cell lines. Cell extracts prepared from the indicated cancer cell lines were analyzed for Bax and Bak expression by immunoblotting.

(H) Inhibition of Bak expression by Bak siRNA in H460 lung cancer cells. H460 cells were transfected with Bak siRNA or control GFP siRNA. After 48 hr, cell lysates were assayed by immunoblotting using anti-Bak antibody.

(I and J) Inhibition of endogenous Bak expression suppresses 3-Cl-AHPC-induced apoptosis. H460 cells were transfected with Bak siRNA or control GFP siRNA. After 36 hr, cells were treated with 3-Cl-AHPC (10^{-6} M) for 24 hr. Apoptosis was determined by DAPI staining (I) and scored (J) as in Figures 5C and 5D.

using epitope-specific anti-Bcl-2 antibodies revealed that Bcl-2 undergoes a conformational change upon Nur77 binding. Given that Nur77/ΔDBD binding reduced epitope availability for an anti-Bcl-2 antibody to the BH3 binding pocket and enhanced the epitope availability for an antibody to the BH3-domain (Figures 7A–7C), it is likely that Nur77 binding induces a rearrangement of the Bcl-2 hydrophobic crevice, resulting in exposure of the otherwise hidden BH3 domain. Such a notion is supported by our findings that the Bcl-2/L137A mutant exhibited enhanced binding to Bak or Bcl-X_L upon Nur77/ΔDBD coexpression, while the Bcl-2 BH3 domain mutant (Bcl-2/Y108K) failed to show such a response (Figures 7D–7E). Based on the observation that Bcl-2 can associate with Bak in Nur77-overexpressing cells, we speculate that Nur77-converted Bcl-2 may similarly function as an agonist of Bak, in addition to an antagonist of Bcl-X_L. Future mutagenesis studies will help to determine whether this hypothesis is correct. However, it is noteworthy that of the 15 known BH3-only proteins in humans and mice, Bid and Bim are the only members that are capable of binding and activating proapoptotic Bcl-2-family proteins Bax and Bak (Korsmeyer et al., 2000; Marani et al., 2002), suggesting a possible role for Bcl-2 when converted to a BH3-displaying killer.

Caspase-mediated cleavage within the loop domain of Bcl-2 converts it into a proapoptotic molecule (Cheng et al., 1997; Grandgirard et al., 1998). However, we do not believe that the Nur77-mediated conversion of Bcl-2 from a protector to a killer involves cleavage of the protein, because we observed no hints of Bcl-2 cleavage in Nur77 overexpressing cells by immunoblotting, and because a mutant of Bcl-2, in which the caspase cleavage site has been mutated (Asp34), remains functional in collaborating with Nur77 to induce apoptosis although with reduced activity (Supplemental Figure S5 available on *Cell* website). Since caspase-mediated or experimental removal of the BH4 domain converts Bcl-2 into a killer, it is conceivable that the BH4 domain of Bcl-2 functions as an inhibitory domain to prevent the exposure of the BH3 domain. Three-dimensional structure of Bcl-2 reveals an extensive interaction between the BH4 domain and the hydrophobic groove (Petros et al., 2001). Therefore, it is tempting to speculate that Nur77, by binding to the loop region in Bcl-2, prevents the inhibitory effect of the BH4 domain, acting as an allosteric regulator to induce a reorganization of the hydrophobic cleft in Bcl-2, leading to exposure of its BH3 domain. This conformational change may be responsible for the conversion of Bcl-2 from an antiapoptotic to a proapoptotic molecule.

Our observations may help explain the paradoxical association of high levels of Bcl-2 protein expression with favorable clinical outcome for patients with several types of cancer, including breast, colon, and nonsmall cell lung cancer (reviewed in Reed, 1996). Possibly, in these tumors, elevated Bcl-2 is a liability, due to conversion of Bcl-2 from an antiapoptotic to a proapoptotic protein through interactions with Nur77 or other proteins. Also, elevated levels of a Nur77-family member are associated with favorable responses to chemotherapeutic agents in patients (Shipp et al., 2002). Interestingly, accumulation of somatic mutations in the region of the *BCL-2* gene encoding the loop domain has also been seen during clinical progression of lymphomas (Tanaka et al., 1992), suggesting the possibility of escape from the conversion mechanism in some types of cancer. Importantly, the discovery of a mechanism for converting Bcl-2 from a protector to a killer might be exploited eventually for developing anticancer drugs that turn overexpression of endogenous Bcl-2, which occurs in approximately half of all human malignancies, into an advantage that promotes tumor cell apoptosis.

Experimental Procedures

(See Supplemental Data available on *Cell* website for detailed procedures)

Bcl-2 siRNAs and Antisense Oligonucleotides

The target siRNA SMARTpools for Bcl-2 and Bak and the siRNA oligonucleotide for Nur77 (5′-CAG UCC AGC CAU GCU CCU dTdT) were purchased from Dharmacon Research Inc. They were transfected into cells according to the manufacturer's recommendations. Bcl-2 antisense oligonucleotide targeting Bcl-2 and negative control oligonucleotides were obtained from Calbiochem.

Nur77/Bcl-2 Interaction Assays

Reporter gene and GST pull-down assays were described previously (Li et al., 2000; Wu et al., 1997). For the mammalian two-hybrid assays, pcDNA-Gal4TAD-Nur77, pcDNA-Gal4TAD-Nur77/ΔDBD, pcDNA-Gal4DBD-Bcl-2/ΔTM, and pcDNA-Gal4DBD-RXRα were cloned and used. For Co-IP assays, HEK293T cells were transiently transfected with various expression plasmids in the presence of caspase inhibitors (zVAD-fmk) to prevent degradation of Nur77 protein due to apoptosis. Antibodies used are: monoclonal mouse anti-GFP (Medical and Biological Laboratories), monoclonal mouse anti-HA (Roche Molecular Biochemicals), monoclonal mouse anti-FLAG (Sigma), monoclonal mouse anti-Myc (Santa Cruz), polyclonal rabbit anti-Nur77 (Active Motif), or monoclonal mouse anti-Bcl-2 (Santa Cruz).

Isolation and Transfection of Human Peripheral Blood Lymphocytes (PBLs)

PBLs were isolated from leukocyte-enriched buffy coats from San Diego Blood Bank by centrifuging on Ficoll-paque Plus (Amersham Pharmacia Biotech). The mononuclear cells were cultured in RPMI containing 10% FBS and 20 mM HEPES. Freshly isolated cells (10^7 cells) were transfected using the human T Cell Nucleofector solution (Amaxa Biosystems) as per the procedure recommended by the manufacturer.

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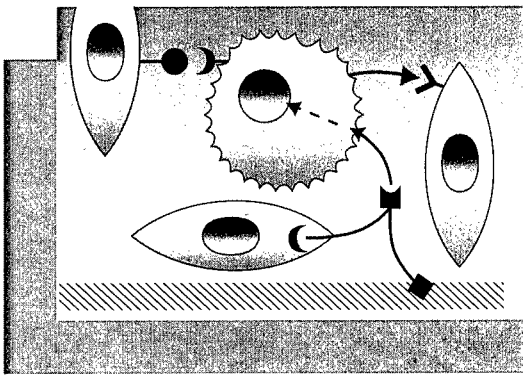

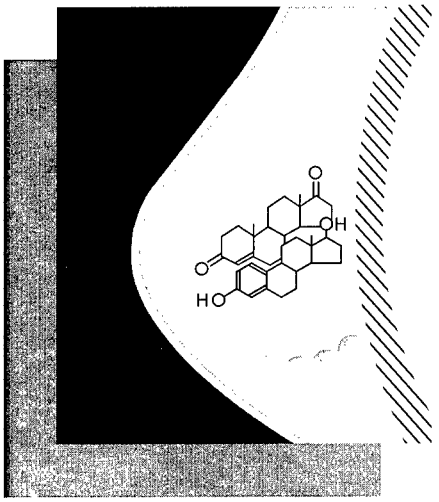

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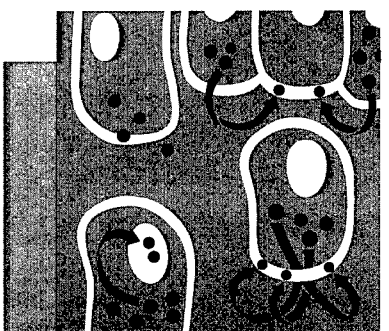

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Endocrine-Related Cancer (2002) **9** 87–102

Vitamin A and apoptosis in prostate cancer

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Abstract

Apoptosis represents an effective way to eliminate cancer cells. Unfortunately, advanced prostate tumors eventually progress to androgen-independent tumors, which are resistant to current therapeutic approaches that act by triggering apoptosis. Vitamin A and its natural and synthetic analogs (retinoids) induce apoptosis in prostate cancer cells *in vitro* and in animal models, mainly through induction of retinoic acid receptor-β (RARβ). Expression levels of RARβ, however, are significantly reduced in hormone-independent prostate cancer cells. Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) that effectively induces apoptosis of both hormone-dependent and -independent prostate cancer cells in a retinoid receptor-independent manner was identified and has drawn a lot of attention in the field. The apoptotic effect of AHPN requires expression of orphan receptor TR3 (also called nur77 or NGFI-B). Paradoxically, TR3 expression is also induced by androgen and other mitogenic agents in prostate cancer cells to confer their proliferation. The recent finding that TR3 migrates from the nucleus to mitochondria to trigger apoptosis in response to AHPN suggests that the opposing biological activities of TR3 are regulated by its subcellular localization. Thus, agents that induce translocalization of TR3 from the nucleus to mitochondria will have improved efficacy against prostate cancer. TR3, therefore, represents an unexplored molecule that may be an ideal target for developing new agents for prostate cancer therapy.

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Introduction

Prostate cancer is the most common cancer diagnosed among men in the United States, accounting for 27.5% of all cancer cases in men. It ranks second after lung cancer as the underlying cause of cancer death in US men. Despite aggressive efforts toward earlier detection and treatment, the mortality rate for prostatic carcinoma has steadily increased. The identification of androgens as the major regulator of prostatic epithelial proliferation offered a target for therapeutic intervention. Androgen ablation by surgical gonadectomy or drug treatments that suppress androgen production and action remain the only effective form of therapy for men with advanced disease. Unfortunately, the median duration of response to androgen ablation is less than 2 years, after which the disease will re-emerge in a poorly differentiated, androgen-independent form, which is often fatal. The lack of therapies for this advanced prostate cancer has contributed significantly to the increased mortality rates, and has resulted in the impetus to develop non-androgen-based therapies.

Vitamin A and its natural and synthetic analogs, retinoids, are one of the most investigated classes of chemopreventive drugs for prostate cancer. Early experiments on mouse prostate explant cultures showed that all-*trans*-retinoic acid (*trans*-RA) could both inhibit and reverse the proliferative effects of chemical carcinogens on prostatic epithelium (Lasnitzki & Goodman 1974, Chopra & Wilkoff 1976). Recent studies have demonstrated that retinoids effectively inhibit the growth of prostate cancer cells *in vitro* and suppress the development of prostate carcinogenesis (Blutt *et al.* 1997, DiPaola *et al.* 1997, Campbell *et al.* 1998, Goossens *et al.* 1999, McCormick *et al.* 1999, Pasquali *et al.* 1999, Richter *et al.* 1999, Sun *et al.* 1999*b*, Urban *et al.* 1999, Webber *et al.* 1999, Kelly *et al.* 2000, Koshiuka *et al.* 2000, Lotan *et al.* 2000, Tanabe 2000, Pili *et al.* 2001). Clinical trials of several retinoids and their combination with other anti-cancer agents have shown significant activities, when retinoids were used in combination with other chemotherapeutic agents, such as interferon-α and paclitaxel (DiPaola *et al.* 1997, 1999, Culine *et al.* 1999, Shalev *et al.* 2000, Thaller *et al.* 2000). Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) (Bernard *et al.* 1992) was found to potently inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate cancer cells. Thus, these small molecules may serve as prototypes for the

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development of new prostate cancer therapeutic and preventive agents. The recent identification of the molecular targets of retinoid action in prostate cancer cells offers opportunities for the development of novel therapeutic strategies.

Vitamin A signaling pathways

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (Zhang & Pfahl 1993, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). RARs and RXRs are encoded by three distinct genes (α, β and γ). In addition, many retinoid receptor isoforms are generated through differential promoter usage, giving rise to a large number of distinct retinoid receptor proteins. To date, there are dozens of receptors which are known to mediate the effect of retinoids. 9-*cis* RA is a high-affinity ligand for both RARs and RXRs, whereas *trans*-RA is a ligand for only RARs. Retinoid receptors belong to a large steroid/thyroid receptor superfamily that mediate the biological effects of many hormones, vitamins and drugs. RARs and RXRs act as transcriptional factors to positively or negatively regulate expression of target genes by binding to their response elements (RAREs) located in promoter regions of the target genes (Fig. 1). The physiological role of RARs and RXRs has been extensively studied by knockout experiments (Kastner *et al.* 1995). Knockout of most of individual RARs activity by homologous recombination appears normal due to redundancy in the function of RARs *in vivo*. However, knockout of RARα and RARγ as well as RAR double knockouts produces defects that resemble the postnatal vitamin A-deficient syndrome and can be prevented by *trans*-RA administration, including keratinizing squamous metaplasia of the prostate gland (Kastner *et al.* 1995).

RXRs form heterodimers with many nuclear receptors including RARs, thyroid hormone receptor (TR), vitamin D receptor and peroxisome proliferator-activated receptor (PPAR) (Zhang & Pfahl 1993, Kastner *et al.* 1995, Mangelsdorf & Evans 1995), thereby mediating diverse endocrine signaling pathways. The function of RARs, however, is more restricted. The role of ligands in the regulation of retinoid receptor function is complex. RAR/RXR is activated mainly through binding of RAR with its ligand, although there are some situations where binding of both the RAR and RXR components with their respective ligands can contribute to the activity of the RAR/RXR heterodimers (Zhang & Pfhal 1993, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). The retinoid binding to RXRs is required for the activation of RXR homodimers and certain RXR heterodimers, such as TR3/RXR and PPARγ/RXR (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). Unliganded retinoid receptors can act as negative transcription factors by binding to the RAREs of retinoid target genes, and recruit receptor corepressors, such as NcoR (Xu *et al.* 1999), leading to histone deacetylation and formation of an inactive chromatin structure preventing transcription. Binding of retinoids to their receptors induces receptor conformational changes that serve as switches by releasing the receptor corepressors and by facilitating the recruitment of receptor co-activators, such as CBP (Xu *et al.* 1999). Several of the co-activator proteins have histone acetylase activity that contributes to the formation of an active chromatin structure and results in the transcription of target genes.

In addition to their direct effects on transcription, liganded RAR can modulate the activity of other transcriptional factors, such as AP-1 (Pfahl 1993). Activated retinoid receptors can inhibit the activity of AP-1, thereby regulating the expression of AP-1 target genes. The inhibition of AP-1 activity is linked to the anti-proliferative effects of retinoids, and appears to be separable from their direct activation of transcription of retinoid-target genes. Synthetic retinoids that specifically inhibit AP-1 activity without activating transcription have been developed (Fanjul *et al.* 1994, Chen *et al.* 1995, Li *et al.* 1996). These AP-1-specific retinoids can inhibit cell proliferation *in vitro*.

Recent evidence indicating that the cytoplasmic action of several hormone receptors represents an important mechanism for regulating their biological function has accumulated. The proapoptotic effect of the orphan receptor TR3 (also known as nur77 and NGFI-B) does not require its transcriptional regulation because TR3 with its DNA-binding domain deleted is still capable of inducing apoptosis (Li *et al.* 2000). In contrast, the cytoplasmic action of TR3, through its mitochondrial targeting, is essential for its apoptotic activity (Li *et al.* 2000). The glucocorticoid receptor was also found to reside on mitochondria (Scheller *et al.* 2000), while differentiation of PC12 phaeochromocytoma cells is accompanied by nuclear export of NGFI-B (Katagiri *et al.* 2000). Estrogen receptors and androgen receptors trigger cell proliferation through their interaction with Src or phosphatidylinositol-3-OH kinase in the cytoplasm (Migliaccio *et al.* 2000, Simoncini *et al.* 2000, Kousteni 2001).

Apoptotic signalings

Apoptosis, also known as programmed cell death, is an evolutionarily conserved and indispensable process during normal embryonic development, tissue homeostasis and regulation of the immune system (Fisher 1994, Steller 1995, White 1996). The apoptotic process can be initiated by several different stimuli, including growth factor withdrawal, DNA damage, deregulation of the cell cycle or ligation of death receptors (Fisher 1994, Steller 1995, White 1996). These different apoptotic stimuli induce diverse early signaling events, which then converge by activating a common central biochemical pathway that is responsible for the execution of apoptosis. Execution of apoptosis is primarily mediated by caspases, a family of cysteine proteases with

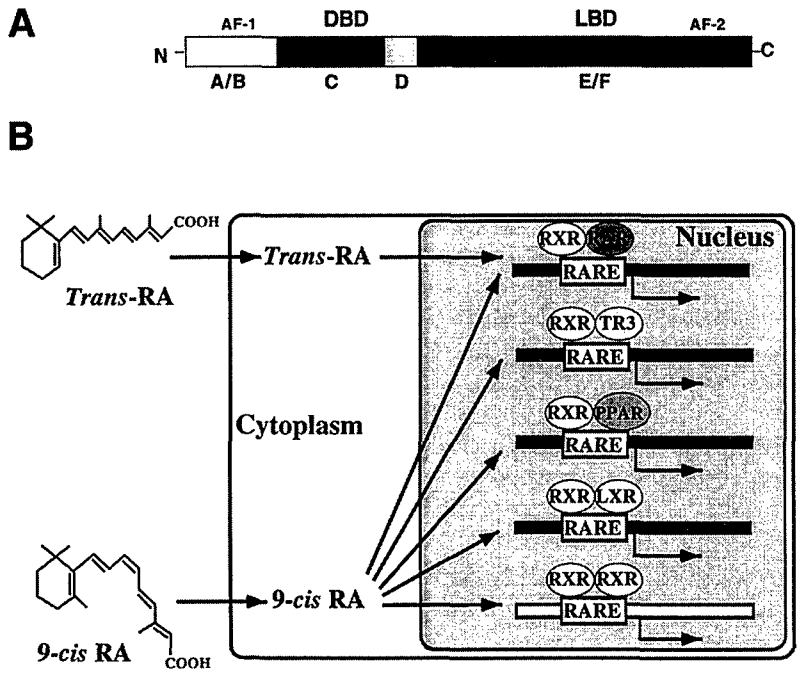

Figure 1 Retinoid signaling. Retinoid receptors are ligand-dependent transcription factors. (A) Schematic representation of retinoid receptor. The receptor may be divided into five regions (A, B, C, D, E and F) based on structural and functional similarities among members of the steroid/thyroid hormone receptor superfamily. DNA binding domain (DBD), ligand binding domain (LBD) and transactivation domains (AF-1 and AF-2) are indicated. (B) Mechanism of action of retinoid receptors. *Trans*-RA or 9-*cis* RA enter cells directly from the circulation, and bind to DNA-bound RAR or RXR, thereby eliciting a transcriptional response.

specificity for aspartic acid residues (Nunez *et al.* 1998, Thornberry & Lazebnik 1998).

There are two distinctly different pathways, the extrinsic and intrinsic pathways, transducing the death signals to caspase-mediated apoptotic machinery (Nunez *et al.* 1998). The extrinsic pathway involves activation of the superfamily of the tumor necrosis factor receptors (TNFR) or CD95 (Fas), by binding to their respective ligands, which in turn recruit procaspase-8 and -10 to membrane-associated signaling complexes, resulting in their activation (Fig. 2). Activation of these upstream caspases is sufficient to directly activate effector caspases such as caspase-3, -6 and -7, or indirectly induce apoptosis by cleaving Bid involved in the release of mitochondrial cytochrome c. The intrinsic pathway is activated directly by various forms of cellular stress that trigger mitochondrial release of cytochrome c into the cytosol. Cytosolic cytochrome c then binds to, and triggers oligomerization of the CED-4 homolog Apaf-1. The resulting 'apoptosome' recruits and activates procaspase-9 which, in turn, recruits and activates effector caspases, such as caspase-3 and possibly caspase-7 (Fig. 2). Additionally, the caspases can be activated by granzyme B, a major serine protease in cytotoxic lymphocyte granules (Shi *et al.* 1992). Once the effector caspases are activated, these enzymes cleave a number of cellular polypeptides leading to disassembly of key structural components of the nucleus and cytoskeleton, inhibition of DNA repair, replication, and transcription, and activation of endonucleases that irreversibly damage the genome (Fisher 1994, White 1996).

Members of the Bcl-2 family are known to modulate apoptosis in different cell types in response to various stimuli (Adams & Cory 1998, Reed 1998). Some members act as antiapoptotic proteins, such as Bcl-2 and Bcl-XL, whereas others function as proapoptotic proteins, such as BAX and BAK. Proapoptotic and antiapoptotic members can heterodimerize and seemingly titrate one another's function. Many Bcl-2 family proteins reside on the mitochondrial outer membrane (Adams & Cory 1998, Reed 1998). Bcl-2 prevents mitochondrial disruption and the release of cytochrome c from mitochondria, while Bax and Bak create pores in mitochondria membranes and induce cytochrome c release. In addition, most proapoptotic proteins antagonize antiapoptotic proteins through heterodimerization with them (Adams & Cory 1998, Reed 1998). Caspase-dependent apoptosis can also be regulated by members of the inhibitors of apoptosis (IAP) protein family. IAPs suppress apoptosis by physically interacting with and inhibiting the catalytic activity of caspases (Deveraux & Reed 1999). In apoptotic cells, the caspase inhibition by IAPs is negatively regulated by a mitochondrial protein Smac/DIABLO, which is released from the

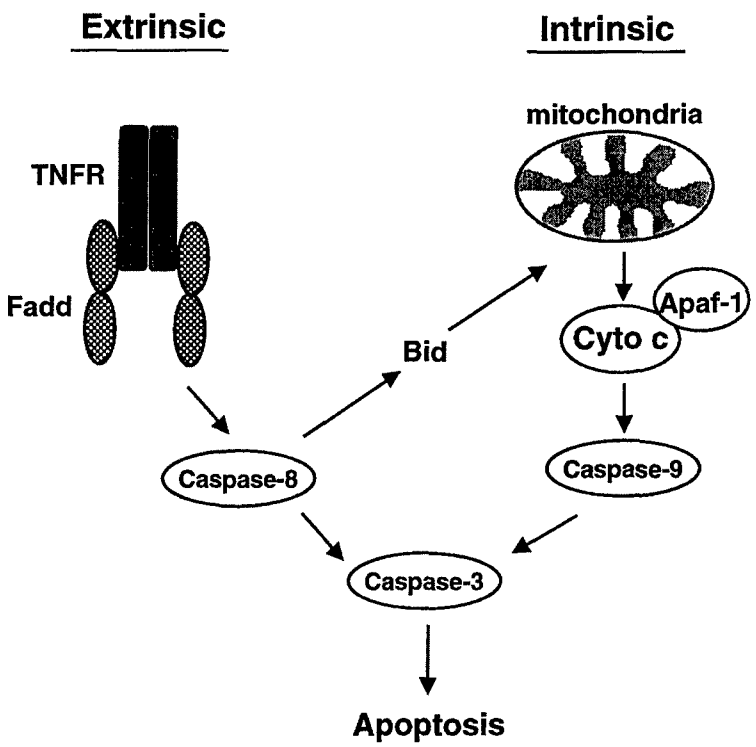

Figure 2 Apoptosis pathways. In the extrinsic pathway, ligation of death receptors activates initiator caspase-8 through the adaptor molecule Fadd. In the intrinsic pathway, cytochrome c (Cyto c) is released from mitochondria in response to a variety of death stimuli and binds to Apaf-1 to activate caspase-9. Active caspase-8 or -9 then activates effector caspases, such as caspase-3, resulting in morphological features of apoptosis. Caspase-8 also activates Bid, which then targets mitochondria to induce cytochrome c release, providing a link between the two pathways.

mitochondrial intermembrane space into the cytosol upon apoptotic stimuli (Du *et al.* 2000, Verhagen *et al.* 2000).

Apoptosis and prostate cancer development

Impaired apoptosis is involved in tumor initiation and progression, since apoptosis normally eliminates cells with increased malignant potential such as those with damaged DNA or aberrant cell cycling (Fisher 1994, Thompson 1995). Most prostate cancer cells have a protracted history of development, suggesting that prostate cancer cells must have evolved various mechanisms to subvert the apoptotic program (Bruckheimer & Kyprianou 2000). Impaired apoptosis signaling and extended cell survival seem to be closely associated with prostate tumor initiation, metastasis and progression to the androgen-insensitive state (Coffey *et al.* 2001). Increased levels of Bcl-2 are associated with emergence of an androgen-independent phenotype and overexpression of Bcl-2 can facilitate multistep prostate carcinogenesis in an animal model (Bruckheimer *et al.* 2000). Proapoptotic Bax contains a polymorphism in an unstable microsatellite causing a frameshift in androgen-independent DU145 cells (Rampino *et al.* 1997).

Recent studies have indicated a crucial role of the PTEN tumor suppressor in the regulation of prostate cancer development. PTEN catalyzes dephosphorylation of phosphatidyl-inositol 3,4,5-trisphosphate and antagonizes signaling pathways that rely on PI3K activity (Wu *et al.* 1998). PTEN is frequently inactivated in primary human prostate cancers, particularly in the more advanced cancers (Ittmann 1998), in human prostate xenografts and in cell lines (Li *et al.* 1997, Vlietstra *et al.* 1998, Whang *et al.* 1998). Release of the negative regulation of the PI3K pathway by PTEN may activate the cell survival kinase Akt during prostate tumor progression (Stambolic *et al.* 1998). Indeed, activated Akt regulates a number of intracellular events implicated in prostate tumor progression and androgen independence. Disruption of PTEN leads to suppression of apoptosis (Stambolic *et al.* 1998), due to inactivation of Bad (Datta *et al.* 1997) or caspase-9 (Cardone *et al.* 1998) by Akt. The disruption can also accelerate cell cycle progression (Sun *et al.* 1999*a*), through suppression of AFX/Forkhead transcription factor activity by Akt (Brunet *et al.* 1999, Kops *et al.*

1999), resulting in inhibition of cell cycle inhibitor p27 expression (Medema *et al.* 2000). The central role played by PTEN has been recently confirmed by the finding that mice with double mutants PTEN(+)/(−)p27(−)/(−) develop prostate cancer at complete penetrance within 3 months from birth (Di Cristofano *et al.* 2001).

Androgen ablation and apoptosis

Androgen withdrawal is the primary choice of therapy for men with advanced prostate cancer, and it generally leads to regression of the disease. It is believed that apoptosis is mainly responsible for the regression of prostate cancer cells (Buttyan *et al.* 2000) and increased levels of apoptosis were indeed observed in human prostate cancer cells after androgen withdrawal (Denmeade *et al.* 1996, Reuter 1997, Montironi *et al.* 1998). However, in the CWR22 human prostate cancer xenograft model it was shown that the regression was due to cell cycle arrest rather than to apoptosis (Agus *et al.* 1999). It remains to be further investigated as to what degree that apoptosis is involved in tumor regression and how the process is regulated.

Progression to androgen independence after androgen-deprivation therapy is a multifactorial process by which cells acquire the ability to proliferate in the absence of androgens. Altered expression of apoptotic-regulatory genes likely plays some role in the development of hormone resistance of prostate cancer (Howell 2000). In the LNCaP prostate tumor model, adjuvant treatment with antisense Bcl-2 oligonucleotides after castration delays progression to androgen independence (Gleave *et al.* 1999). Androgen-independent prostate cancer cells also show resistance to apoptosis induction by chemotherapeutic agents and radiotherapy (Bruckheimer & Kyprianou 2000, Szostak & Kyprianou 2000). Overexpression of Bcl-2 and Bcl-XL is found in many androgen-independent cell lines and may be responsible for resistance to apoptosis (Bruckheimer & Kyprianou 2000, Coffey *et al.* 2001, Li *et al.* 2001), and antisense Bcl-2 oligonucleotides sensitize prostate cancer cells to the apoptotic effect of chemotherapeutic agents (Leung *et al.* 2001).

Retinoids and prostate cancer apoptosis

Growing evidence suggests that induction of apoptosis is a major mode of cell death in response to most cancer chemopreventive and chemotherapeutic agents (Fisher 1994, Thompson 1995, Bruckheimer & Kyprianou 2000). Retinoids exert potent apoptotic effects both in development and in cancer cells (Nagy *et al.* 1998). Retinoid-induced teratogenesis is associated with craniofacial malformations due to excessive apoptosis in the region (Sulik *et al.* 1988), while the limb malformations induced by retinoids are also associated with excessive cell death in the apical ectodermal ridge (Sulik & Dehart 1988). Retinoids regulate the development of the central nervous system in part through their apoptotic effect (Alles & Sulik 1990, 1992).

Induction of apoptosis by retinoids has been observed in various prostate cancer cells *in vitro* and *in vivo*. *Trans*-RA induces apoptosis of normal and malignant epithelial prostate cells (Pasquali *et al.* 1999), and it strongly enhances the apoptotic effect of docetaxel in DU-145 and LNCaP prostate cancer cells (Nehme *et al.* 2001). The combination of *trans*-RA and organic arsenical melarsoprol synergistically induces apoptosis of DU-145 and PC-3 cells *in vitro* and in immunodeficient mice (Koshiuka *et al.* 2000). The synthetic retinoid N-(4-hydroxyphenyl) retinamide (4HPR) is known to induce apoptosis in various malignant cells (Nagy *et al.* 1998). 4HPR also induces apoptosis of androgen-dependent and -independent cells (Sun *et al.* 1999*b*, Webber *et al.* 1999). The combination of 13-*cis* RA and phenylbutyrate synergistically induces apoptosis of several human and rodent prostate carcinoma cell lines (Pili *et al.* 2001).

The molecular mechanisms by which retinoids induce apoptosis of prostate cancer cells remain largely unknown. Induction of apoptosis of prostate cancer cells by several retinoids appears to be associated with down-regulation of Bcl-2 expression (DiPaola & Aisner 1999, DiPaola *et al.* 1999, Pasquali *et al.* 1999, Nehme *et al.* 2001), induction of insulin-like growth factor-binding protein-3 (IGFBP-3) (Goossens *et al.* 1999) and tissue transglutaminase (Pasquali *et al.* 1999), an enzyme that accumulates in cells undergoing apoptosis. Interestingly, RXRα was found to interact with IGFBP-3, and IGFBP-3-induced apoptosis was abolished in RXRα-knockout cells. It is likely that RXRα/IGFBP-3 interactions modulate the effects of IGFBP-3 on apoptosis (Liu *et al.* 2000).

RARβ and retinoid responses

The involvement of retinoid receptors in mediating proapoptotic effects of retinoids is complex, since some retinoids may act in a retinoid receptor-independent manner. However, many studies have suggested a crucial role of RARβ in the modulation of retinoid-induced apoptosis of prostate cancer cells. RARβ is up-regulated during apoptosis induced by the combination of phenylbutyrate and 13-*cis* RA in human and rodent prostate carcinoma cell lines and prostate tumors in the xenograft model (Pili *et al.* 2001), suggesting that RARβ expression may mediate the growth-inhibitory effect of retinoids. RARβ was also induced during *trans*-RA-induced apoptosis of prostate cancer cells (Pasquali *et al.* 1999). The expression of RARβ in 4HPR-treated prostate tissue was slightly higher than in the placebo-treated group (Lotan *et al.* 2000). Interestingly, introduction of RARβ in RARβ-negative prostate cancer cells resulted in increased sensitivity to the growth-inhibitory effect of retinoids and vitamin D (Campbell *et al.* 1998).

The role of RARβ in mediating the growth-inhibitory effect of retinoids was also demonstrated in many different types of cancer cells, including breast, lung, ovarian, neuroblastoma, renal cell, pancreatic, liver, and head and neck (Nervi *et al.* 1991, Li *et al.* 1995, Hoffman *et al.* 1996, Liu *et al.* 1996, Kaiser *et al.* 1997, Xu *et al.* 1997*b*, Campbell *et al.* 1998, Ferrari *et al.* 1998, Li & Wan 1998). Expression of RARβ in RARβ-negative cancer cells restored *trans*-RA-induced growth inhibition and apoptosis, whereas inhibition of RARβ expression in RARβ-positive cancer cells abolished *trans*-RA effects (Li *et al.* 1995, Liu *et al.* 1996, Li & Wan 1998). In addition, transgenic mice expressing RARβ antisense sequences showed increased incidence of lung tumors (Berard *et al.* 1996), whereas suppression of RARβ expression was responsible for diminished anti-cancer activities of retinoids in animals (Wang *et al.* 1999). The expression of RARβ decreases as breast cells become progressively more malignant (Xu *et al.* 1997*a*), suggesting that loss of RARβ may lead to breast cancer development. Furthermore, up-regulation of RARβ is associated with a positive clinical response to retinoid in patients with premalignant oral lesions (Lotan *et al.* 1995).

The involvement of RARβ is also implicated by the finding that its expression mediates prostatic ductal branching morphogenesis in response to retinoids (Aboseif *et al.* 1997). The expression of RARβ and RXRβ was significantly reduced in malignant prostates compared with normal prostates (Lotan *et al.* 2000). In contrast, RARα, RARγ, RXRα and RXRγ were expressed in both normal and prostate tumor tissues (Lotan *et al.* 2000). RARβ was also selectively lost in DU-145 and PC-3 androgen-independent prostate cancer cells lines while RARα, RARγ and RXRα were well expressed (Campbell *et al.* 1998, Sun *et al.* 1999*b*). These observations suggest that loss of RARβ is associated with prostate carcinogenesis. The fact that reduced RARβ was observed in the normal tissue adjacent to the tumor suggests that this change is an early event in prostate carcinogenesis (Lotan *et al.* 2000). Similar changes were also observed in head and neck cancer (Xu *et al.* 1994).

How RARβ exerts its potent tumor-suppressive effects remains to be elucidated. A recent study demonstrated that RARβ can potently inhibit AP-1 activity (Lin *et al.* 2000*b*) and induce apoptosis of various cancer cells. The proapoptotic effect of RARβ was implicated in the finding that the expression of RARβ in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (Dolle *et al.* 1989, Mendelsohn *et al.* 1991, Ruberte *et al.* 1991, Kochhar *et al.* 1993, Soprano *et al.* 1993*a*,*b*). In our previous study, we observed that *trans*-RA-induced apoptosis in ZR-75-1 breast cancer cells is mediated by RARβ (Liu *et al.* 1996). Inhibition of RARβ activity by the expression of RARβ anti-sense RNA reduced the number of apoptotic cells, whereas *trans*-RA-induced apoptosis was only observed in hormone-independent cells when RARβ was introduced and expressed in the cells (Liu *et al.* 1996).

The mechanism that causes loss of RARβ in prostate cancer is not clear. It is unlikely that lack of RARβ expression is due to structural abnormalities of the RARβ gene (Gebert *et al.* 1991), but possibly because of changes in transcription. Expression of RARβ is highly induced by *trans*-RA through a RARE (βRARE) present in its promoter (Hoffmann *et al.* 1990, Sucov *et al.* 1990, de The *et al.* 1990), which is activated by RAR/RXR heterodimers in response to retinoids (Zhang *et al.* 1992*a*). Vitamin A serum levels are lower in patients with prostate cancer (Reichman *et al.* 1990). In addition, prostate cancer tissues have five to eight times less *trans*-RA than normal prostate or benign prostate (Pasquali *et al.* 1996). Reduced levels of retinoids in prostate cancer tissue may contribute to loss of RARβ expression. Interestingly, RARβ cannot be induced by exogenous retinoids in androgen-independent prostate cancer cells, despite expression of RARs and RXRs in these cells (Sun *et al.* 1999*b*). Similar observations were also made in other cancer, such as lung cancer, cells which express RARs and RXRs, but fail to express RARβ in response to retinoids (Zhang *et al.* 1994). These observations argue against the involvement of reduced retinoid levels in inhibiting RARβ expression, and also demonstrate that expression of RARs and RXRs is not sufficient to render RARβ expression responsive to *trans*-RA. Thus, factors other than RARs and RXRs are required for the effect of *trans*-RA on inducing RARβ expression, and these may be lost in cancer cells. Recently, we found that expression of the orphan receptor COUP-TF is positively correlated with RARβ induction and growth inhibition by *trans*-RA in various cancer cell lines and it is underexpressed in many RARβ-negative cancer cell lines (Wu *et al.* 1997*b*, Lin *et al.* 2000*a*). Further studies demonstrated that COUP-TF is required for *trans*-RA to induce RARβ expression, growth inhibition and apoptosis in cancer cells (Lin *et al.* 2000*a*). The effect of COUP-TF is likely due to its transactivation of the RARβ promoter through its binding to a DR-8 element present in the promoter, resulting in enhanced interaction of RARα with its co-activator CBP (Lin *et al.* 2000*a*). Thus, COUP-TF induces RARβ promoter transcription by acting as an accessory protein for RARα to recruit its co-activator. Whether lack of COUP-TF expression is responsible for loss of RARβ in androgen-independent prostate cancer cells remains to be illustrated. Methylation of the RARβ promoter was recently reported to contribute to RARβ inactivity (Sirchia *et al.* 2000), suggesting a possibility of hypermethylation of the RARβ promoter in prostate cancer cells.

The anti-cancer effects of conventional retinoids appear to be limited to androgen-dependent prostate cancer cells, whereas the more aggressive, androgen-independent prostate

cancer cells are refractory (Campbell *et al.* 1998). Loss of RARβ induction by *trans*-RA may be responsible for diminishment of *trans*-RA activities in androgen-independent prostate cancer cells. Induction of RARβ by classical retinoids, such as *trans*-RA, is mediated by activation of RAR/RXR heterodimers which bind to the βRARE (Zhang *et al.* 1992*a*). Unfortunately, this pathway appears to be impaired in androgen-independent prostate cancer cells. It is therefore important to identify alternative pathways that activate the RARβ promoter. Recent studies have demonstrated that RXR-selective retinoids represent promising agents for the prevention and treatment of cancer. 9-*cis* RA has demonstrated significant anti-proliferative and/or differentiating activity in *in vitro* models of breast cancer (Anzano *et al.* 1994, Rubin *et al.* 1994, Gottardis *et al.* 1996*b*), leukemia and lymphoma (Gottardis *et al.* 1996*b*), lung cancer (Guzey *et al.* 1998), and head and neck cancer (Giannini *et al.* 1997). Its activity was also observed in prostate cancer cells (Blutt *et al.* 1997, McCormick *et al.* 1999). Combination of 9-*cis* RA and 1,25-dihydroxyvitamin D3 synergistically inhibited the growth of LNCaP (Blutt *et al.* 1997, McCormick *et al.* 1999). McCormick *et al.* (1999) conducted a chemoprevention study to evaluate the activity of 9-*cis* RA as an inhibitor of prostate carcinogenesis in animals, and observed that continuous dietary administration of 9-*cis* RA before MNU administration reduced cancer incidence in the dorsolateral+anterior prostate. Similarly, the dosage levels of 9-*cis* RA reduced the incidence of cancer in all accessory sex glands (McCormick *et al.* 1999). RXR-selective retinoids were more effective than *trans*-RA at inhibiting mammary carcinogenesis in animals (Anzano *et al.* 1994), and RXR-selective retinoid LGD 1069 inhibited the growth of established breast tumors (Gottardis *et al.* 1996*a*, Bischoff *et al.* 1998).

How RXR ligands effectively inhibit the growth of cancer cells has not been established. Through its binding to RXR, RXR ligands may indirectly influence a wide range of functions, which are regulated by other nuclear receptors that heterodimerize with RXR (Zhang & Pfahl 1993, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). In our previous studies (Wu *et al.* 1997*a*), we observed that inhibition of cancer cell growth by RXR-selective retinoids was associated with induction of RARβ expression in estrogen-independent MDA-MB231 cells and lung cancer cells (Wu *et al.* 1997*a*), suggesting that induction of RARβ expression contributes to the growth-inhibitory effects of these retinoids. Furthermore, we observed that their effect on RARβ induction is in part mediated through TR3/RXR heterodimers which bind to the βRARE (Wu *et al.* 1997*a*). Thus, RXR ligands may exert their potent anti-cancer activity through inducing RARβ expression in cancer cells that are resistant to classical retinoids (Fig. 3). Thus, specific ligands for the RXR receptor may have significant activity as inhibitors of carcinogenesis in the prostate, whereas retinoids whose binding is limited to RAR may be inactive.

AHPN and its analogs: potent apoptotic inducers of prostate cancer cells

The sensitivity of prostate cancer cells to apoptosis-inducing effects of retinoids diminishes during the progression of prostate tumors. Androgen-independent derivatives of LNCaP cells were more resistant than their parental androgen-dependent LNCaP cells to apoptotic effects of *trans*-RA. In addition, malignant prostate cancer cells showed resistance to radiotherapy and chemotherapy. This has been the major challenge in the therapy of prostate cancer. Thus, retinoids capable of inducing apoptosis of advanced malignant prostate cancer cells are expected to be suitable agents for prostate cancer treatment.

Recently, a new class of synthetic retinoids related to AHPN (also called CD437) (Bernard *et al.* 1992) has been found to potently inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate carcinoma cells (Liang *et al.* 1999, Lu *et al.* 1999, Li *et al.* 2000, Sun *et al.* 2000). When the growth-inhibitory and apoptosis-inducing effects of *trans*-RA and AHPN were compared in androgen-dependent and -independent prostate cancer cell lines, AHPN significantly inhibited the growth and induced apoptosis of androgen-independent prostate cancer cell lines, while *trans*-RA had little effect on these cells (Sun *et al.* 2000). A synthetic retinoid, CD-271, which is related to AHPN and selectively activates the RARγ subtype in a given context, also shows increased anti-proliferative activity against prostate cancer cells over *trans*-RA (Lu *et al.* 1999). Interestingly, AHPN was more effective in killing androgen-independent cells such as DU-145 and PC-3 than the androgen-dependent LNCaP cells (Sun *et al.* 2000). Thus, AHPN may be representative of a novel class of compounds suitable for treatment of androgen-independent prostate cancer. AHPN was also identified to be a potent apoptotic inducer in many different types of cancers, including lung (Sun *et al.* 1997, 1999*c*,*d*,*e*, Adachi *et al.* 1998*b*, Li *et al.* 1998), cervical (Oridate *et al.* 1997), ovarian (Langdon *et al.* 1998), melanoma (Schadendorf *et al.* 1995, 1996), leukemia (Hsu *et al.* 1997, Gianni & de The 1999, Mologni *et al.* 1999) and neuroblastoma (Meister *et al.* 1998). The apoptotic effect of AHPN is independent of retinoid receptor expression, indicating that its activity is not restricted by lack of RARβ in prostate cancer cells.

Orphan receptor TR3: a regulator of both survival and apoptosis of prostate cancer cells

AHPN-induced apoptosis may involve p53-dependent and -independent as well as caspase-dependent and -independent pathways (Adachi *et al.* 1998*a*, Fontana *et al.* 1998, Hsu *et al.* 1999, Marchetti *et al.* 1999, Zhang *et al.* 1999, Zhang

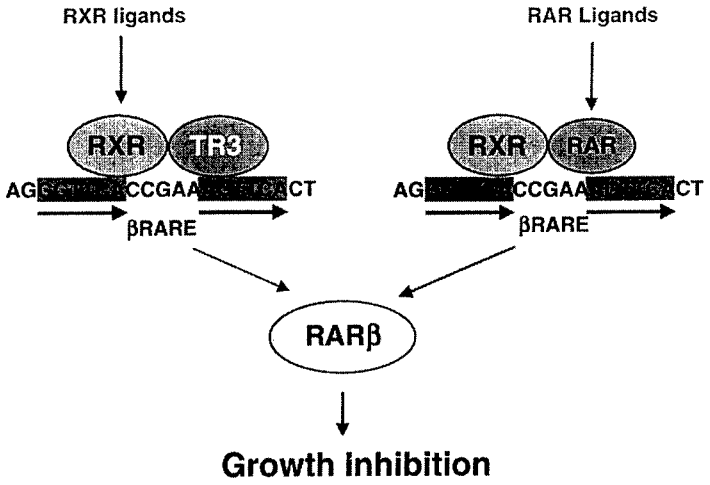

Figure 3 Signaling pathways for RARβ induction. βRARE in the RARβ promoter is essential for induction of RARβ by retinoids. The βRARE can be activated by RAR/RXR heterodimer in response to RAR ligands. Alternatively, it can be activated by RXR ligands through a TR3/RXR heterodimer that also binds to the βRARE.

2000). Expression of a variety of apoptosis-associated genes, such as cJun, cFos, c-Myc, p21, Bcl-2, Bax, DR4, DR5 and Fas can be regulated by AHPN in a cell type-specific manner. Their role in AHPN-induced apoptosis, however, remains to be determined. We have recently demonstrated that the expression of TR3 is required for AHPN-induced apoptosis in human prostate cancer cells (Li *et al.* 2000). TR3 message was also highly induced by AHPN in LNCaP cells (Li *et al.* 2000). The apoptotic effect of the AHPN analog MM11453 was completely abolished in LNCaP cells stably expressing TR3 antisense RNA (Li *et al.* 2000).

TR3 (Chang & Kokontis 1988, Hazel *et al.* 1988, Milbrandt 1988) is an immediate–early response gene whose expression is rapidly induced by a variety of growth stimuli, including growth factors, phorbol ester and cAMP-dependent pathways (Chang & Kokontis 1988, Hazel *et al.* 1988, Milbrandt 1988, Fahrner *et al.* 1990, Wilson *et al.* 1993, Crawford *et al.* 1995, Lim *et al.* 1995). It is also an orphan member of the steroid/thyroid/retinoid receptor superfamily (Zhang *et al.* 1992*b*, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). Like other immediate–early growth response genes, such as c-myc and c-jun, TR3 plays a role in controlling cell proliferation and mediating apoptosis (Bravo 1990, Herschman 1991). TR3 is rapidly induced during apoptosis in immature thymocytes and T-cell hybridomas (Liu *et al.* 1994, Woronicz *et al.* 1994). Overexpression of a dominant negative TR3 protein (Woronicz *et al.* 1994) or inhibition of TR3 expression by antisense TR3 inhibits apoptosis in thymocytes (Liu *et al.* 1994), whereas constitutive expression of TR3 results in massive apoptosis (Xue *et al.* 1997).

TR3 plays a critical role in regulating both proliferation and apoptosis of prostate cancer cells. Levels of TR3 are dramatically induced by androgen (Uemura & Chang 1998) and epidermal growth factor (Li *et al.* 2000) in LNCaP prostate cancer cells. Interestingly, TR3 is also rapidly induced in LNCaP cells in response to apoptotic stimuli, including AHPN (Li *et al.* 2000), calcium ionophore, etoposide (VP-16) (Uemura & Chang 1998, Li *et al.* 2000) and phorbol ester (Young *et al.* 1994, Li *et al.* 2000) and in the ventral prostate of animals by androgen ablation (Uemura & Chang 1998). Expression of TR3 antisense RNA significantly inhibits apoptosis induced by these agents (Li *et al.* 1998, Uemura & Chang 1998). Because of its potent effects in regulating cellular proliferation and apoptosis, TR3 may play a role in the development or progression of prostate cancer. In fact, TR3 is more highly expressed in prostate cancer areas than in adjacent normal or benign prostate hypertrophic tissue (Uemura & Chang 1998). TR3 is also highly expressed in lung cancer cell lines (Wu *et al.* 1997*b*). The role of TR3 in cancer development is further indicated by the finding that TR3 is involved in a chromosomal translocation identified in extra-skeletal myxoid chondrosarcoma (Labelle *et al.* 1995, 1999).

How TR3 exerts opposing biological activities was poorly understood. Similar to other members of the steroid/thyroid/retinoid receptor superfamily, it was believed that TR3 functioned in the nucleus as a transcriptional factor to regulate gene expression necessary to alter the cellular phenotype in response to various stimuli. TR3 response elements (NBRE or NurRE) have been identified (Wilson *et al.* 1991, Philips *et al.* 1997). In addition, TR3 can heterodimerize with RXR (Forman *et al.* 1995, Perlmann & Jansson 1995, Wu *et al.* 1997*a*) and COUP-TF (Wu *et al.* 1997*b*). The observations that over-expression of TR3 in cancer cells confers retinoid resistance by modulating transcriptional

regulation of retinoids (Wu *et al.* 1997*b*) and that the TR3 fusion protein identified in extra-skeletal myxoid chondrosarcoma is about 270-fold more active than the native receptor in transactivation (Labelle *et al.* 1995, 1999) suggests that TR3 may mediate cell proliferation through its transcriptional regulation.

Much less was known about the mechanism by which TR3 functions to regulate apoptosis. TR3 might be involved in the apoptotic process by regulating expression of certain apoptosis-associated genes (Liu *et al.* 1994, Woronicz *et al.* 1994, 1995, Weih *et al.* 1996, Cheng *et al.* 1997). Unfortunately, no comprehensive characterization of its target genes was achieved. By using a variety of approaches, we recently demonstrated that TR3-dependent apoptosis of LNCaP prostate cancer cells does not require its DNA binding and transactivation, but is associated with translocation of this protein from the nucleus to mitochondria, where it resides on the outer mitochondrial membrane and induces cytochrome c release (Li *et al.* 2000). These results reveal a novel mechanism by which a nuclear transcriptional factor translocates to mitochondria to initiate apoptosis (Fig. 4). Translocation of TR3 between the nucleus and the cytoplasm represents a new mechanism for cross-talk between different signaling pathways (Fig. 4). This exciting finding, together with the observations that TR3 is associated with cancer cell proliferation by acting as a nuclear transcriptional factor, demonstrates that the opposing biological activities of TR3 are regulated by its subcellular localization. These data suggest a new approach of eliminating prostate cancer cells by inducing cytoplasmic localization of TR3. AHPN analogs and other agents that specifically induce TR3 mitochondrial localization will effectively induce apoptosis of prostate cancer cells that express TR3. Interestingly TR3 is induced by androgen or growth factors through nuclear action of TR3. Thus, AHPN and related analogs may be potent inhibitors of androgen and growth factor action in prostate cancer cells.

Prospective

Induction of apoptosis is an effective way to eliminate cancer cells. The acquisition of resistance toward apoptosis during prostate tumor progression is perhaps the major obstacle in the treatment of prostate cancer. Retinoids inhibit the growth and induce apoptosis of prostate cancer cells *in vitro* and prevent prostate carcinogenesis in animals, suggesting that retinoids are promising agents for the prevention and treatment of human prostate cancer. However, the apoptotic effect of classical retinoids diminishes in androgen-independent prostate cancer cells, and clinical trials using conventional retinoids have not demonstrated significantly beneficial effects. Loss of RARβ may contribute to retinoid resistance in advanced prostate cancer cells. Alternative approaches to induce RARβ expression may render prostate cancer cells sensitive to apoptotic effects of retinoids. *In vitro* and animal studies have suggested that RXR ligands are effective inhibitors of prostate carcinogenesis and they are capable of inducing RARβ expression through alternative approaches, such as TR3/RXR heterodimers. Elucidation of their mechanisms of action will provide valuable information, allowing design and identification of a new generation of synthetic retinoids that are likely to be more effective in the prevention and treatment of prostate cancer.

Synthetic retinoids related to AHPN effectively induce apoptosis of both androgen-dependent and -independent prostate cancer cells, indicating that these retinoids represent a new class of drugs that have therapeutic value for the treatment of prostate cancer. The clinical potential of this class of retinoids and their new generation needs to be explored.

Modern biology has suggested that cancer drug discovery based on molecular differences between tumor and normal cells is a new and feasible approach. With an improved understanding of apoptotic processes in prostate cancer cells, many potential new targets for therapy can be discovered. The illustration that orphan receptor TR3 mediates the apoptotic effect of AHPN analogs in prostate cancer cells suggests that TR3 is an ideal target for cancer drug development. Levels of TR3 are induced by androgen and growth factor in prostate cancer cells as well as by androgen ablation and may be necessary to support proliferation of prostate cancer cells. Thus, TR3 can mediate opposing biological activities, cell death and survival (Fig. 4). The unique property of TR3 provides an excellent opportunity to develop novel drugs targeted at TR3. Agents such as AHPN and its analogs that specifically induce mitochondrial localization of TR3 will convert TR3 from a cancer cell-promoting (adverse effect) to a cancer cell apoptosis-inducing (beneficial effect) molecule.

Cellular localization of TR3 defines its biological function. How TR3 is translocated from the nucleus to the cytoplasm and targets mitochondria in response to apoptotic stimuli is unclear. This information is essential for developing retinoids that induce mitochondrial localization of TR3. The fact that TR3 mitochondrial targeting is regulated by various stimuli, including TPA, calcium ionophore and growth factors (Li *et al.* 2000), which are known to act through membrane signaling pathways involving various kinases and phosphatases, suggests that phosphorylation of TR3 may play a crucial role in regulating TR3 subcellular activities.

The observation that TR3 can heterodimerize with RXR (Forman *et al.* 1995, Perlmann & Jansson 1995, Wu *et al.* 1997*a*) suggests that RXR and its ligands are likely involved in the regulation of TR3-dependent apoptotic pathways. This is supported by previous observations that RXR and its ligand 9-*cis*-RA inhibit activation-induced apoptosis of T-cells and thymocytes (Yang *et al.* 1993, 1995*a*,*b*, Bissonnette *et al.* 1995, Szondy *et al.* 1998), in which TR3 plays a role (Liu *et al.* 1994, Woronicz *et al.* 1994, 1995). RXR, through its heterodimerization with TR3, may be required

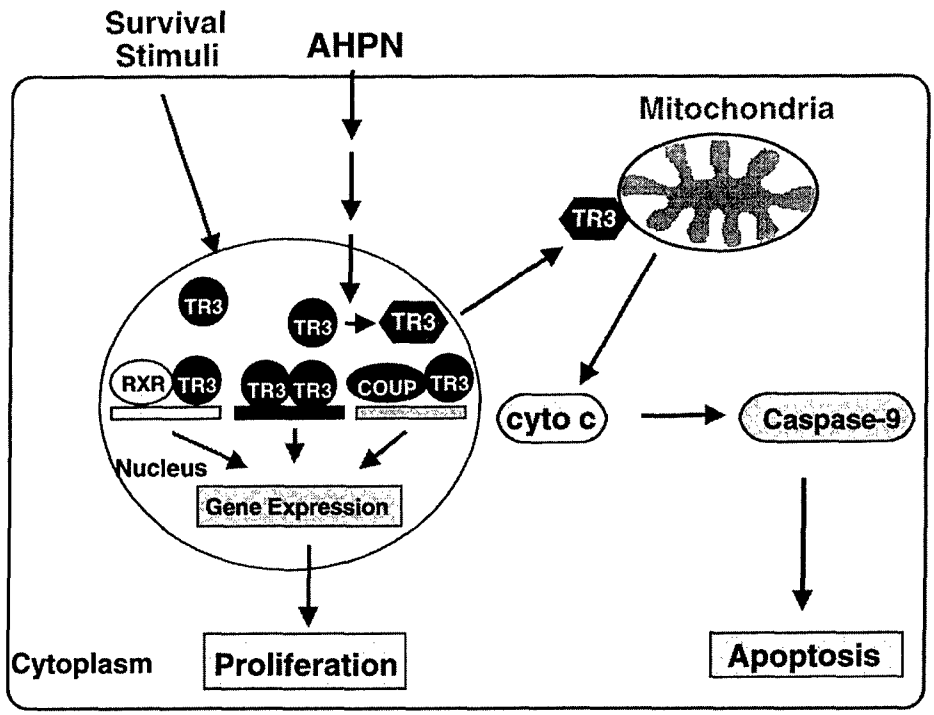

Figure 4 TR3-dependent cell survival and cell death pathways. TR3 induced by survival stimuli, such as growth factors, functions in the nucleus through either its homodimerization or heterodimerization with RXR or COUP-TF to regulate expression of genes involved in cell proliferation. In contrast, TR3 induced by death stimuli, including AHPN, may undergo a conformational change, which is required for its export to the cytoplasm, where it resides on mitochondria. On mitochondria, TR3 regulates mitochondrial activities, resulting in release of cytochrome c (cyto c) into the cytosol.

for cytoplasmic localization of TR3 or for its mitochondrial targeting. Illustrating the molecular mechanisms by which RXR and its ligands regulate TR3-dependent apoptotic pathways in prostate cancer cells will provide additional modes to regulate apoptosis of prostate cancer cells and new treatment approaches for prostate cancer.

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